THE USE OF METABOLOMICS TO PREDICT CHEESE FLAVOUR DEVELOPMENT

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Asal HajNajafi

Master of Food Engineering

(Universiti Putra Malaysia)

School of Science

College of Science, Engineering and Health

RMIT University

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DECLARATION

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and ethics procedures and guidelines have been followed. I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

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List of Conference presentations

Parts of this thesis have been presented as both poster and oral presentations, at appropriate scientific conferences as outlined below.

Oral presentations

- **HajNajafi A. (2018),** "The use of metabolomics in cheese flavour", Australian Centre for Research on Separation Science (ACROSS), winter symposium, Monash University, Australia.

Poster presentations

- **HajNajafi A. (2019),** Zisu B., Gill H., and Jones O.A.H., "The Use of metabolomics in cheese flavour", Proteomics & Metabolomics Victoria Symposium, Melbourne, Australia.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ASN	Acid Soluble Nitrogen
CV	Cross Validation
D_2O	Deuterium oxide
DVB/ CAR/ PDMS	Divinylbenzene/Carboxen/Polydimethylsiloxane
GABA	γ-aminobutyric acid
GC-MS	Gas Chromatography-Mass Spectroscopy
HCA	Hierarchical Cluster Analysis
HNMR	High Resolution Nuclear Magnetic Resonance
MLR	Multiple Linear Regression
NIR	Near Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance
NSLAB	Non-Starter Lactic Acid bacteria
OLS	Ordinary Least Squares
PCA	Principal Component Analysis
PCR	Principal Component Regression
PCO	Principal Coordinates Ordination
PLS	Partial Least Squares Regression
PLS-DA	Partial Least Squares Regression Discriminant Analysis
PRIMER	Plymouth Routines in Multivariate Ecological Research
R^2	Root mean square
RMS Err	Root Mean Square Error
SEP	Standard Error of Prediction
SPME	Solid Phase Micro Extraction

Chapter 1 GENERAL INTRODUCTION

1.1 Cheese

Cheese is an important dairy based food product that is made in many forms all over the world. In all varieties the flavour develops during the ripening process, during which time complex biochemical changes take place, leading to the formation of a range of biomolecules, including amino and organic acids and numerous volatile compounds, which all contribute to the overall flavour.

The ripening and associated flavour development of hard cheeses, such as cheddar, is very well defined. In contrast the ripening process (and associated development of flavours) of soft cheese is not well studied and not well understood, making it difficult for manufactures to produce such cheese with uniform flavour qualities. Likewise, it is difficult to identify procedures that cause off-flavour compounds or even estimate if/when they will occur during ripening. Problems during ripening are often only discovered at the end of the maturation process, which can sometimes take months or even years.

The loss of a high value product due to off flavours after an extended maturation time can have substantial, adverse economic effects to the manufacturer. There is therefore an interest in the cheese industry to find new and faster ways to detect and predict issues in soft cheese maturation as early as possible. Alternative techniques are needed and the study of metabolites, or metabolomics, could be one of these new methods.

Metabolomics is a relatively new field of ‘‘omics’’ research concerned with the high-throughput identification and quantification of small molecule (<1500 Da) metabolites. These molecules can include a range of endogenous and exogenous chemical entities such as, amino acids, nucleic acids, carbohydrates, organic acids, vitamins, polyphenols, alkaloids, minerals and just about any other chemical that can be synthesized or utilised by a given cell, tissue or organism.

Metabolomics has been widely applied to many disciplines, including microbiology, human health, drug discovery and development, and food and beverage analysis. Within food science metabolomics has recently been applied for monitoring the quality, processing, safety, and

microbiology of both raw materials and final products to improve food quality. Dairy products, as a major sector of the food industry, are also being explored using metabolomic techniques. However, while cheeses account for up to 50% of total dairy products relatively few attempts have been made to profile cheese via metabolomics and those studies that have been performed did not connect metabolites to cheese quality and flavour during development. For this to occur it helps to have a good understanding of cheese flavour formation and how it is currently evaluated.

1.2 Flavour evaluation

It has been recognized for many years (probably since the market began) that sensory quality is one of the most crucial aspects of sales and marketing in the dairy industry (Bodyfelt et al., 1988; Singh et al., 2003). The consistency of cheese quality and flavour is thus a great concern for cheese manufactures since bad flavour and/or significant variation between batches of the same product can negatively affect consumer value.

Sensory analysis is composed of various tools and tests that can be utilised for evaluating sensory food properties (Whetstine and Drake, 2008). Characterization of the sensory attributes of cheeses is traditionally performed via tasting assays, for which people are trained to be able to classify and describe the different flavours involved. It is also important to note that some compounds that do not taste nice are still important. A good example is Butyric acid. This compound smells quite bad to most people and does not impact an especially desirable flavour. However, when it reacts with ethanol, it forms ethyl butyrate, which smells like ripe pineapple - and thus is desirable. Such complex interactions are hard to capture with standard flavour analysis.

Descriptive sensory analysis is performed to determine the flavour profile of a food component and uses trained analysts to taste and describe a product. Although subjective, this technique is considered ideal for identifying different flavours in a product and also distinguishing amongst differing, but similar, products such as wine or cheese (Murray et al., 2001). This sort of analysis can also be used in combination with instrumental analysis to gather a more complete picture of flavour. Indeed, a detailed understanding the chemical composition of a product, and

individual compounds specific contribution to the overall flavour is required in order to better understand flavour formation (Drake et al., 2007).

Instrumental techniques can be used for extraction and analysis of aroma-active compounds in various foods. Usually it is found that aroma compounds are present at very low concentrations. Isolation and concentration procedures are then required so that chemicals of interest can be detected (Qian and Reineccius, 2003). This can be difficult to achieve hence a careful choice of methodology needs to be taken in order to characterize flavour compounds accurately. By selecting an appropriate sensory and instrumental technique, a detailed study of flavours can be undertaken.

1.3 Aims and objectives

The overall aim of this project is to link advances in analytical science and metabolomics to improve the flavour analysis of soft cheese (with a focus on Camembert) so that these techniques can be used as a more reliable management tool in cheese manufacturers' strategic decision-making processes.

The objectives of this study are to;

(i) Identify and characterise primary and secondary metabolites produced during ripening of soft cheese (with a focus on Camembert cheese) and determine where they originate.

(ii) Determine the relationship between metabolites, odour properties and volatile chemical compounds.

(iii) Discover metabolites that could serve as biomarkers (predictors) of cheese quality and flavour.

(iv) To combine the data from points i-iii to develop metabolic models of cheese ripening and provide systematic techniques to help cheese manufactures to efficiently monitor the quality and flavour of their products.

Novel analytical techniques such as nuclear magnetic resonance (NMR), fourier transform infrared spectroscopy (FTIR), gas and liquid chromatography – mass spectrometry (GC- and LC-MS) and chemometrics approaches for data analysis (principal component analysis and partial least squares-discriminant analysis) will be employed for the above aims.

1.4 Final outcome

The investigation of cheeses using metabolomic techniques will provide new scientific information that explains the variation in quality and flavour of cheese. On the completion of this project our knowledge in cheese flavour profile will be increased and the knowledge out of this project will significantly benefit the dairy industry and dairy science in general.

New and improved knowledge in flavour chemistry could lead to consistent delivery of desired or targeted cheese flavour profile, enhancement of specific aroma attributes in cheese flavour profile to match consume taste profile and to develop of novel product options in soft cheese.

Chapter 2 LITERATURE REVIEW

2.1 Introduction

Cheese is an important fermented dairy based product that is made in many forms all over the world in more than 1000 varieties (Fox and McSweeney, 2004; Sandine and Elliker, 1970). Compared to many dairy products, cheese has significant advantages as a food product due to its fat content, solid matrix and buffering capacity (Rodrigues et al., 2011).

Cheese is divided in two categories, natural, and processed (Ochi et al., 2012). Natural cheese is prepared using cow's milk. Rennet is first added to milk, followed by lactic acid bacteria as a starter culture. The particular lactic acid bacteria that used, the methodology utilised, the area where the cheese is produced, the food the cow ate during milk production and the ripening time can all cause differences in the final product. Biochemical changes are observed in the cheese during ripening resulting in the development of a specific flavour, aroma and texture (McSweeney, 2004b) and this variety in cheese is vital for it as a product (Ochi et al., 2012).

By contract, processed cheese is produced by commuting, melting and emulsifying one or multiple natural cheeses. A consistent of soft uniform mixture using heat, mechanical shear and (usually) emulsifying salts (Ochi et al., 2012) is usually evident. The final cheese is obtained using mass production techniques and while the final product is more consistent, it is usually thought to be inferior in taste and health to naturally produced cheese. A natural cheese flavour is one of the main criteria that effect consumer acceptance of processed cheese quality.

Traditional tools used for the sensory evaluation of cheese quality are dependent upon grading and judging. These methods work well for testing the generic quality of the cheese when many samples need to be rapidly assessed. However, grading and judging results do not reflect consumer preferences. Therefore, these cannot be considered ideal tools for cheese flavour research. Currently, the most powerful sensory tool in cheese flavour research is descriptive sensory analysis (Ochi et al., 2012).

Various studies have been conducted on cheese flavour characteristics using descriptive sensory analysis (Drake et al., 2001; Heisserer and Iv, 1992). Sensory evaluation is generally found to be time-consuming and costly. Therefore, much research has been concentrated on the isolation/detection of particular, 'special' compounds in cheese that affect particular sensory characteristics. The aim of such research is to use these compounds to replace time-consuming and costly sensory analysis with cheaper and faster experimental evaluations and generally focuses on odour-active compounds volatile compounds. Such compounds are associated with nutty flavour in Cheddar (Avsar et al., 2004), and the pungent flavour of Gouda-type cheeses (Van Leuven et al., 2008). Some researchers have also focused on the water-soluble flavour compounds in hard cheeses such as Cheddar (Andersen et al., 2010), Gouda (Toelstede and Hofmann, 2008) and Comte (Salles et al., 1995) as well as in goat's milk based cheese (Engel et al., 2000). Whatever systems are employed, the complexity in the formation of cheese creates significant challenges for the cheese industry to apply simply scientific strategies. Consequently, grading by humans, based on abilities, knowledge and experience is currently, still essential for quality control during cheese production (Ochi et al., 2012). This approach is common in the food industry but is in contract to many other industries which rely more on advanced analytical methods for consistency of product (the e.g. pharmaceutical industry).

Notwithstanding the above, the molecular understanding of food is now rapidly increasing with the introduction of techniques such as analytical chemistry and specialised methods such as metabolomics to ascertain what causes unique flavour, texture, aroma and colour in foods (Piras et al., 2013). Since it is difficult to control cheese quality by using a conventional approach focused on one or several target compounds, metabolomics, which looks at and quantifies as many compounds as possible, is a potentially interesting method (Ochi et al., 2012). The application of metabolomics has the potential to facilitate major advances in our understanding cheese quality and the components of the cheese metabolome. It would also allow the application of this understanding to help control and monitor quality during cheese making.

2.2 Manufacture of cheese

The manufacturing process is very similar for almost all varieties of cheese, and only minor modifications are made to achieve desired characteristics in different cheese. The processing steps, their control and effect on cheese quality have been explained in detail by several authors (Fox et al., 2000a; Lawrence et al., 2004) and so are not explored further here. The general process for the production of cheese is shown in Figure 1.1. Milk (pasteurized or unpasteurized) is mixed with starter culture, rennet in a vat. The starter cultures reduce the pH of the milk by producing lactic acid from lactose. Rennet, which is more active at lower pH values, then coagulates the protein (casein). Optionally, calcium chloride ($CaCl_2$) may be added (at around 0.01%) since calcium plays an important role in coagulation of milk with rennet (Farkyea and Fox, 1990)

To achieve desirable organoleptic qualities, most rennet cheeses are ripened before consumption. After the coagulum is formed it is cut and then matured. After draining the whey (the liquid remaining after milk has been curdled and strained) the curd is matted and then milled. In dry-salted cheeses like Cheddar, salt (1 to 3%) is sprinkled and mixed thoroughly. Salt improves flavour, controls fermentation, and reduces the moisture content. The curd is then pressed into blocks, packaged and aged/ripened. Ripening period varies from 2 months to 2 years or more depends on the cheese (Singh and Cadwallader, 2008). Complex physical, chemical, and microbiological changes take place during the ripening process, leading to the development of characteristic texture and flavour of the cheese. These processes include a sequence complex of biochemical pathways, which can be classified into proteolysis, lipolysis and lactose/ lactate degradation. The maturation types depend on the shelf life, storage temperature, cheese composition (most importantly moisture content and salt percentage) and enzymes activity type and also the microflora (Farkyea and Fox, 1990).

The manufacture of rennet-coagulated cheeses can be classified into two parts i) the conversion of milk into curd, which is essentially complete within 24 hours ii) and ripening of the curd, which can take weeks, months or even years (Singh and Cadwallader, 2008).

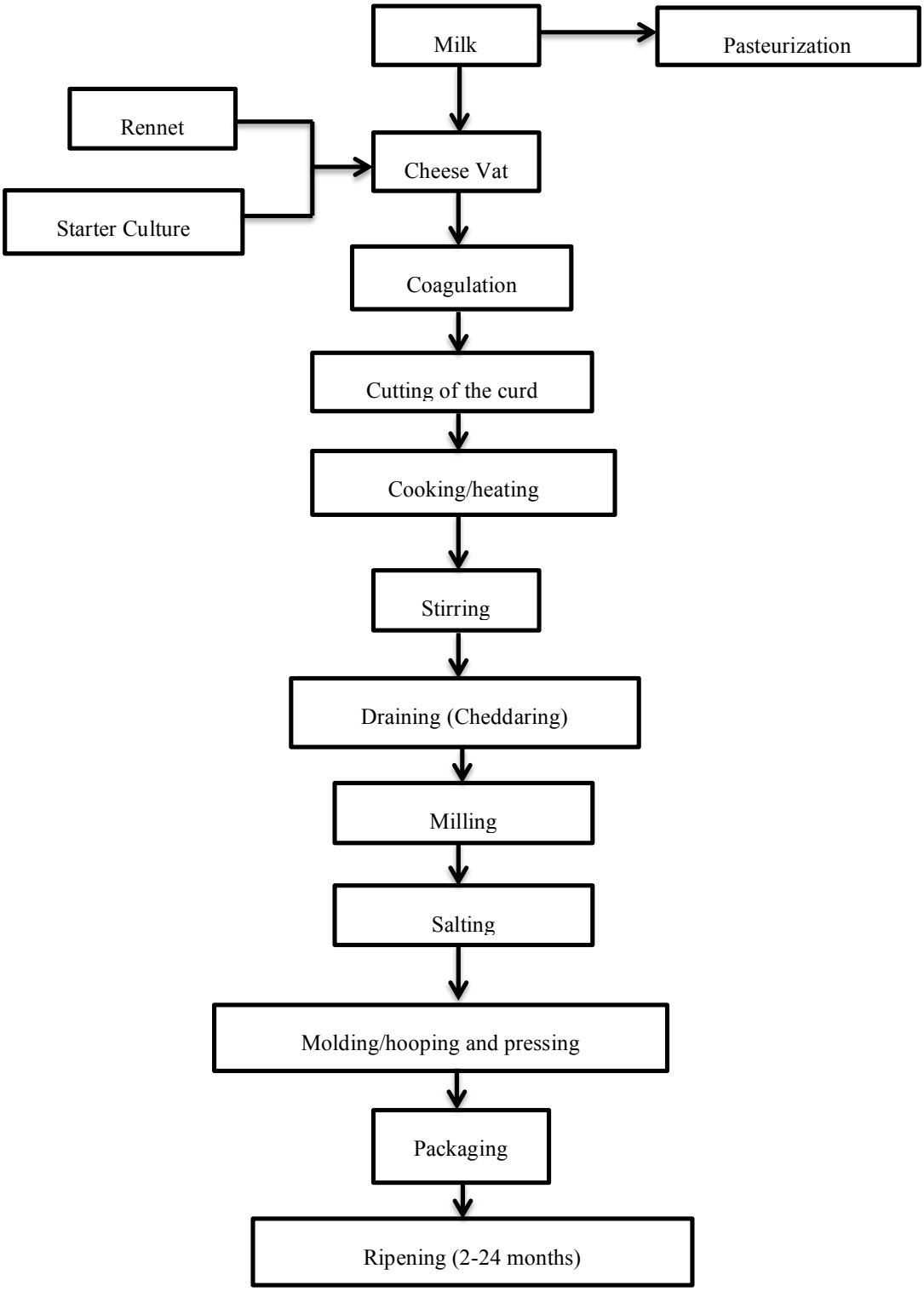


Figure 2-1 General descriptions of steps involved in the cheese manufacture. Adopted from Singh and Cadwallader (2008)

2.3 Cheese ripening

The bacteria engaged in cheese production and cheese maturation are subdivided into two classes. Firstly, bacteria added to the milk by careful selection of starter cultures by the manufacturer and secondly, non-starter lactic acid bacteria (NSLAB) which are naturally present in the milk (Awad et al., 2007). The cheese industry's need to give the customers healthy cheeses with high organoleptic properties in a proper maturation time has led to the new techniques, such as the use of adjunct cultures. Adjunct cultures are special strains of microorganisms that are added to milk that help to improve cheese sensory quality. With the exception of natural sources of non-starter lactic acid bacteria, adjuncts are particularly chosen and deliberately introduced to improve the cheese milk microflora to enhance the general quality of final cheese product (Soda et al., 2000). Starter and non-starter bacteria are both essential for acid formation that quickens the coagulation of milk and also helps in whey suppression. Furthermore, they supports effects on the texture of final cheese products (Law, 2001). For example the effects of *enterococci* bacteria in making cheese are associated with hydrolysing fat and protein in milk to assist in curd formation (El-Din et al., 2002). *Enterococci* bacteria are now specifically recommended as a starter culture in Cebreiro cheese production (Centeno et al., 1996; Litopoulou-Tzanetaki et al., 1993) even though the product is traditionally a raw milk cheese.

2.4 Biochemical reaction during production and maturation of cheese

There are various biochemical reactions that happen in cheese maturation. Firstly, the metabolism of residual lactose, lactate and citrate, and also lipolysis, and proteolysis. Secondly, metabolism of fatty acids and amino acids that cause the development of volatile flavours (Harper and Kristoffersen, 1956; McSweeney, 2004b). Elaborate discussions of all the biochemical changes during hard cheese ripening have been published (Collins et al., 2003a; Collins et al., 2004; Curtin and McSweeney, 2004; Marilley and Casey, 2004; McSweeney, 2004b; McSweeney and Fox, 2004; Upadhyay et al., 2004; Yvon and Rijnen, 2001a) but not soft cheese ripening.

2.4.1 Glycolysis and metabolism of lactose, lactate and citrate

Usually cheese contains lactic acid L (+positive) and D (-negative) isomers. The relativity of the proportion of D-isomer is dependent upon the type of starter culture used and on various

ripening factors. The D (-) content of lactic acid in various types of cheese can be very different. In fresh cheese it can be 4-14% and in ripened cheese in may be 10-50% of the total. Most of the remaining lactose is lost as whey during whey drainage. From a quality standpoint, complete fermentation of lactose is essential to avoid the growth of undesirable secondary organisms in the cheese which could affect flavour or be detrimental to health (McSweeney, 2004b). Any remaining lactose is converted to D-lactate and L-lactate by NSLAB and racemization, respectively (Fox et al., 2000b; McSweeney, 2004a). In most cheese varieties, the conversion of lactose to lactic acid mainly happens after the curds have been put in moulds. Lactate can change via several pathways as shown in Figure 2.2.

The extent and the rate of acidification during production has a significant influence on texture through the demineralization of casein micelles, which in turn influences proteolysis (Fox et al., 1990). The oxidation can take place by using lactic acid bacteria (LAB) in cheese to convert to acetate, ethanol, formic acid and also carbon dioxide at a rate dependent on oxygen availability (Fox et al., 2000b). Other processes include breakdown by *Propioni bacterium* spp. and formation of propionate, acetate, water and carbon dioxide, as well as *Penicillium* spp yeasts to form carbon dioxide and also *Clostridium* spp. to produce butyric acid and hydrogen.

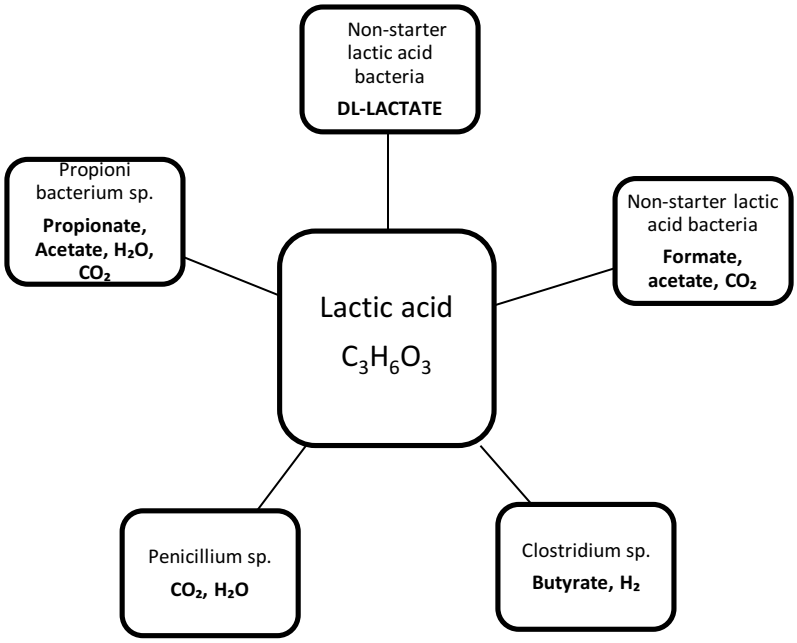


Figure 2-2 Pathways for metabolization of lactate during ripening. Adopted from Fox and McSweeney (2004)

Citrate is also considered to be a vital compound for the flavour development in cheese making (Fox and McSweeney, 2004; McSweeney, 2004a). The main source of citrate in cheese is milk. Bacteria can metabolize citrate to acetoin, acetate, butanediol, and diacetyl which all have their own flavour notes (Cogan and Hill, 1993; Palles et al., 1998).

2.4.2 Lipolysis and metabolism of free fatty acids

Lipolysis is one of the most important biochemical events during cheese maturation as it develops the generation of multiple flavour compounds (Collins et al., 2003a; Collins et al., 2004; McSweeney and Sousa, 2000). Its effect on the flavour of blue cheese and hard Italian cheese has been studied extensively and the mechanism of lipid breakdown and pathways of flavour production were reviewed in detail recently by Collins et al. (2004).

Breakdown of fats in cheese is primarily by hydrolysis of triglycerides by the enzyme lipase. Oxidation is very limited due to low oxygen availability. Lipases and esterases in cheese may come from milk, rennet paste, starter cultures, secondary microorganisms, NSLAB, or exogenous lipase preparations (Collins et al., 2004).

Milk is also low in short-chain fatty acids, which have low flavour thresholds. Formation of these short-chain free fatty acids by lipolysis is thus a desirable process in soft cheese (McSweeney, 2004a). However, contribution of lipolysis to flavour and quality of hard cheeses such as Cheddar is limited. Excessive lipolysis is in fact considered undesirable in this case as it ruins the flavour (McSweeney, 2004a). Free fatty acids also serve as precursors to the formation of several volatile flavour compounds which are more prevalent in soft cheeses than hard (Collins et al., 2003a,b; McSweeney and Sousa, 2000). An overview of lipolysis and catabolism of fatty acids is shown in Figure 2.3.

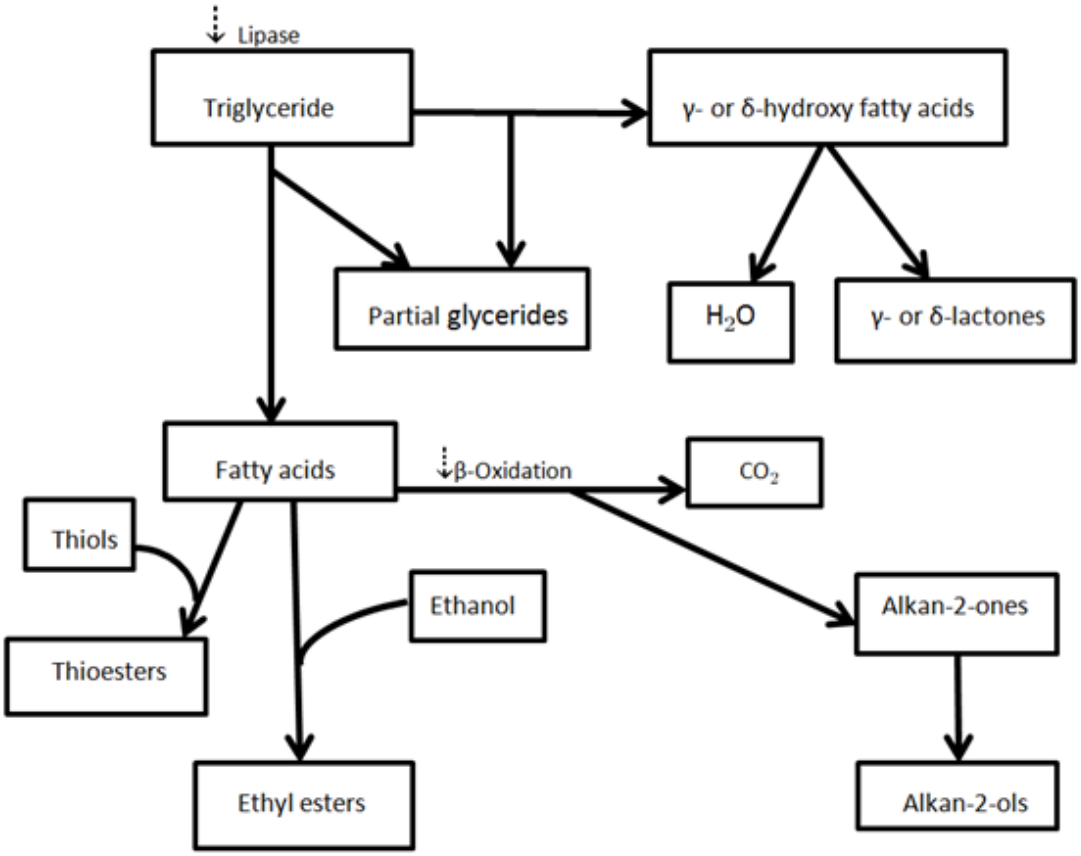


Figure 2-3 Pathways for lipolysis and catabolism of free fatty acids. Adopted from Collins et al (2004)

2.4.3 Proteolysis and metabolism of fatty acids

Proteolysis is one of the most vital processes that occurs during cheese ripening because the catabolism of amino acids. Upadhyay et al. (2004) compiled some of the published work on proteolysis and amino acid breakdown in a comprehensive review as have others over the years (Aston et al., 1983; Curtin and McSweeney, 2004; Fox, 1989; Fox et al., 2000b; McSweeney and Sousa, 2000; Yvon and Rijnen, 2001a). A summary of the proteolytic reactions and amino acid catabolism is shown in Figure 2.4.

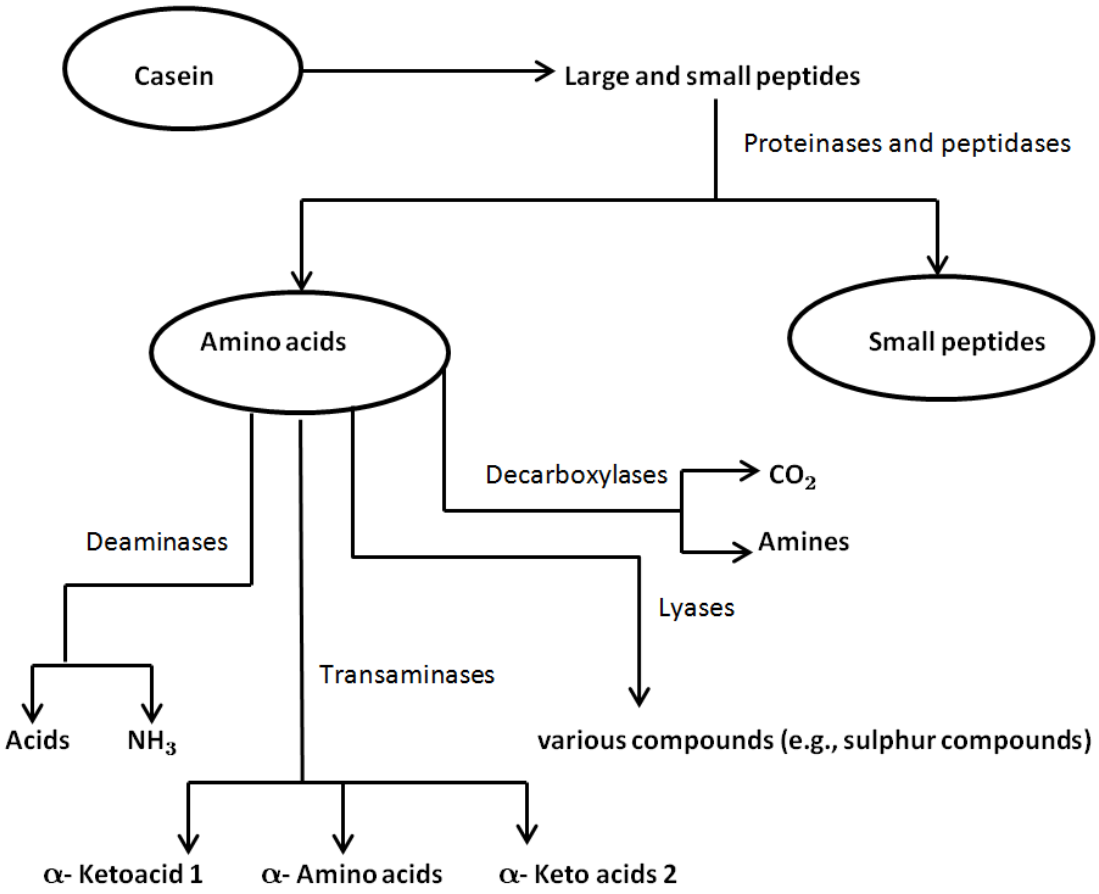


Figure 2-4 Overview of proteolysis and acid catabolism during cheese ripening. Adopted from McSweeney (2004b)

Proteolysis contributes significantly to the flavour and texture of the cheese. It also plays a significant role in flavour development during ripening. This is because many of the short peptides produced during proteolysis are flavourful and because formation of several important volatile flavour compounds is due to the release of amino acids via proteolysis. Furthermore, proteolysis enables protein bound compounds to be released from the cheese matrix. Proteolysis also softens the texture of cheese by 1) hydrolysing the casein matrix, 2) decreasing water activity via the formation of the new carboxylic acid and amino groups from hydrolysis (changes in the water binding), and 3) increasing the pH by formation of ammonia from amino acids during proteolysis.

Proteinases and peptidases are main proteolytic agents in cheese and, aside from rennet may come from the milk, coagulants, starter LAB and NSLAB, moulds (in mould ripened cheeses), Gram + bacteria (in smear type cheeses) and secondary microorganisms that come in from the

air (Upadhyay et al., 2004). Milk enzymes, and to some extent cathepsins, also contribute to proteolysis. Enzymes from the microorganisms may also influence the degree and the rate of proteolysis.

The first stage of proteolysis is the results of enzymatic action which breakdown casein into large and medium-sized peptides which are then broken down further to small peptides and amino acids. The procedures involved in the changes of amino acids to volatile flavour compounds are not fully known but some studies have been undertaken. Curtin and McSweeney (2004) published a summary of the various studies in cheese flavour development by catabolism of amino acids. There are two main pathways: 1) aminotransferase or lyase activity and 2) deamination or decarboxylation. Lyase activity causes the formation of α-ketoacids. The α-ketoacids are further broken down to flavour compounds such as hydroxyl acids, aldehydes, and carboxylic acids. α-Ketoacids from methionine, large amino acids (leuine, isoleucine, and valine) or aromatic amino acids (phenylalanine, tyrosine and tryptophan) are precursors to many volatile flavour compounds (Yvon and Rijnen, 2001a).

In contrast, methionine generally forms volatile sulphur compounds. Methanethiol, which contributes to the characteristic flavour of Cheddar cheese at low concentrations, is formed from the degredation of methionine (Curtin and McSweeney, 2004; Weimer et al., 1999). In addition, bacterial lyases can metabolize methionine to α-ketobutyrate, methanethiol, and ammonia (Soda et al., 1983; Tanaka et al., 1985). Volatile flavour compounds such as Benz aldehyde, phenyl acetate, phenyl ethanol, phenyl lactate form by aminotransferase aromatic amino acids catabolism. Deamination reactions result in α-ketoacids and ammonia, which help to develop the flavour of cheese. However, biogenic amines can cause flavour defects via decarboxylation of amino acids.

2.5 Role of the starter culture during ripening

The starter culture bacteria are generally responsible for the production of lactic acid and metabolism of citric acid. Lactic acid acts as a preservative which also contributes to the acidic flavour of some cheeses. Starter bacteria are also involved in protein degradation (together with the rennet and milk enzymes), and also contribution to the breakdown of the diglycerides

formed from the milk triglycerides by milk lipoprotein lipase. These bacteria are also responsible for the degradation of hippuric acid to benzoic acid, which again acts as a natural preservative (Cogan and Hill, 1993).

2.6 Cheese Flavour

Flavour is the feeling generated in the mouth of the consumer, identified primarily by the senses of taste and smell, and also by the mouth's overall receptors of pain, tactile sensation, and temperature. Flavour refers to the sum of the sensory attributes and is one of the three primary sensory properties that are important in food choice, adoption, and intake (Hassan et al., 2013).

Cheese flavour is one of the most fundamental factors for consumer choice and acceptance (Delgado et al., 2010). The particular flavour of a cheese variety is the effect of a complicated equilibrium between volatile and non-volatile chemical compounds, derived from milk fat, protein and carbohydrates formed during the maturation process (Delgado et al., 2010). Each product has a specific characteristic and structure of components (Plutowska and Wardencki, 2007). Different fresh cheese curds all have bland and mainly comparable flavours and aroma. Flavour compounds specific for each variety are formed during the maturation process (McSweeney, 2004b; McSweeney and Sousa, 2000). The flavour of new produced cheese, which is prepared to be consumed instantly after production, is the outcome of the starter bacteria activity and mainly due to diacetyls and potentially acetaldehydes. The flavour of aged cheese is the outcome of the starter bacteria interaction, the milk enzymes, rennet maturation and associated lipases, and secondary microbial flora (Urbach, 1997b).

The metabolism of lactose and lactate, lipolysis and proteolysis are the main pathways for the creation and development of flavour compounds in cheese (Delgado et al., 2010). Depending on variety, microflora and requirements for maturation, lactate can be metabolised and breakdown by a number of processes to different compounds that add to the cheese flavour (Delgado et al., 2010). The second pathway produces fat-derived compounds with low flavour thresholds developed by lipolysis and lipid oxidation processes, such as free fatty acids, esters, lactones and ketones (Delgado et al., 2010). Lastly, casein proteolysis offers a variety of small and medium sized peptides and free amino acids that likely only contribute to the background

flavour of most types of cheese. However, free amino acids are the essential components for a variety of poorly-understood catabolic processes that generate flavour compounds (McSweeney and Sousa, 2000).

In summary, in general, during cheese maturation, Flavour compounds are developed with glycolysis, lipolysis and proteolysis pathways due to milk enzymes, rennet and micro-organisms (Fox and McSweeney, 2004). Proteolysis is the most significant biochemical pathway for the developing of flavour and texture in most type cheeses (Visser, 1993). A well-balanced breakdown of curd proteins like casein into smaller peptides and amino acids is important for formation of an appropriate cheese flavour and aroma (Singh et al., 2003). The proteolytic enzymes from lactic acid bacteria (LAB) play a key role in casein and peptide degradation, leading to free amino acid formation (Randazzo et al., 2007). The transformation of each amino acid results in the creation of particular volatile compounds in cheese (Randazzo et al., 2007). The conversion of leucine and isoleucine for example, leads in the production and development of 3-methylbutanal and 2-methylbutanal, respectively, which have been observed to be the main flavour components in several cheese types (Engels, 1997; Pederson et al., 1999).

Table 1.1 shows major flavour compounds of different cheese types. The work on monitoring the development of flavour in dairy products, such as cheese, can concentrate on two areas: a general increase in all main flavour components (improved/faster cheese maturation) or an increase in some key flavours. It might well lead to a new types of cheese with an acknowledged flavour growth, but can also lead to a known flavour imbalance, which called off-flavour (Ayad et al., 2000).

Metabolite type	Gouda	Cheddar	Camembert	Swiss-type
Amino acid	3-Methylbutanal 3-Methylbutanol Methanethiol Dimethylsulphide (DMS) 2-Methylpropanol Dimethyltrisulphide(DMTS)	3-Methylbutanal Isovaleric acid Methional Methanethiol DMDS DMTS	3-Methylbutyrate 3-Methylbutanal Methional Methanethiol DMS Benzaldehyde Phenylacetaldehyde	Methional 3-Methylbutanal Skatole
Sugar	Diacetyl	Propionic acid Diacetyl	2,3-Butanedione	Propionic acid Diacetyl
Fat	Butyric acid Butanon Hexanal Pentanal	Butyric acid Acetic acid 1-Octen-3-one Butanone	1-Octen-3-ol Butyric acid 1-Octen-3-one 2-Undecalactone γ-Decalactone	
Rest and combined pathways	Ethyl butyrate Limonene	Ethyl butyrate Ethyl hexanoate	Phenylethyl acetate	Ethyl butyrate Ethyl hexanoate Ethyl-3-methylbutanoate Phenylethyl acetate

Table 2-1 Important flavour components in four types of cheese (Smit & Engels, 2005)

2.7 Camembert cheese

2.7.1 Background and characteristics

One of the more common of the multiple types of soft cheeses is Camembert. It is interesting as it has a surface ripened by mould and the milk is slightly cultured before rennin is added. The coagulated milk is cut, moulded, salted and aged for two to five weeks. A mat of mould mycelia grows on the surface during the early stages of ripening. The texture of the cheese becomes almost fluid and the flavour is nutty with a slight ammonia-like taste once it is mature (Edward, 1990).

2.7.2 Starter cultures

Much of the research done on Camembert has focused on the identification of the microbial flora, microbial contamination and the adaptation of new processing technologies. The ripening process has been extensively tested using various coagulants, starter organism, direct set curd, enzymes and other models of cheese ripening.

Single or multiple strains of *Streptococcus lactis* and/or *S. cremoris* are used to inoculate the cheese milk for Camembert. These starter cultures are mesophilic and grow well at 20°C to 25°C. These organisms are gram-positive, non-motile, catalase negative cocci. These lactic acid starters are homo-fermentative, produce no gas from glucose and produce D-lactic acid (Kosikowski and Mistry, 1977). The mould strains, used in the Camembert ripening, are classified as *Fungi Imperfecti*. These mould strains are referred to as *Penicillium candidium*. At the beginning of this century, the accepted classification for the mould used in the surface ripening of Camembert and Brie was *P. camemberti* a white mould with a greyish tint common to the Camembert cheese ripening rooms of France. Most Camembert produced in factories today use spores of *P. caseicolum* strains as inoculum because it gives a more snowy white appearance to the final product. Commercially, this mould is commonly referred to as *P. candidium*.

In Camembert made from goat milk or on the farm *P. album* is used because of its more rapid growth rate. *P. candidium* and *P. album* grouped together as *P. camemberti (Samson et al., 1977)*. The mould strains used commercially for spore inoculation were isolated from the ripening rooms in factories in the Camembert region of France (hence the name). Strain selection has been performed using colour of the mycelium, salt tolerance, pH range, enzymatic activity and resistance to contamination. *Geotrichum candidium* may also be found in association with *P. caseicolum*. It quickens the ripening of Camembert and gives the cheese a yeastier flavour (Kosikowski and Mistry, 1977).

2.7.3 Process

In Camembert manufacture, the whole milk is first pasteurised to kill the pathogenic and spoilage organisms. The temperature of the pasteurised milk is adjusted to 35° C. A mixed lactic acid bacteria culture, which produces the acidity needed for proper coagulation, is added to the milk. Mould spores may also be added to the milk at this time. A coagulant, such as

rennin or another suitable milk clotting enzyme is then also added. The inoculated milk is incubated at 35° C until the coagulum is sufficiently firm to cut. The coagulated milk is cut into one-inch cubes. The cubes are ladled into 4-5 inch hoops and drained on reed mats. At 5, 10 and 15 hours of draining, the cheese is turned. After 20 hours of draining, the cheese is removed and brined for one hour in a saturated sodium chloride solution. After brining, it is drained for 1/2 hour. Next the cheese is placed into a curing room set at 13° to 16° C and 95% to 98% relative humidity. Additional mould spores may be inoculated onto the cheese surface after each turning and/or after brining. After 4 and 8 days, the cheese is turned on the shelf to facilitate even mould growth. After 10 to 12 days, the cheese is wrapped to limit further mould growth and water evaporation but the action of the mould enzymes continue inward. The curd softens as it ages and the flavour becomes mushroomy or mould like. The cheese is ready for distribution and consumption after two to five weeks of ripening depending on the preference of the consumer. To slow down further ripening, the temperature of the cheese can be reduced to 4° C (Kosikowski and Mistry, 1977).

2.8 Metabolomics and flavour analysis techniques

The molecular understanding of food is rapidly increasing with the introduction of metabolomics to see what causes unique flavour, texture, aroma and colour in foods (Piras et al., 2013). With regard to food science, metabolomics has been performed recently to monitor the quality, handling and production, safety and microbiology of both raw materials and final products in order to enhance the health and confidence of consumers and to meet current and future requirements in agricultural and food science (Cevallos-Cevallos et al., 2009; Rodrigues et al., 2011).

Metabolomics intends to incorporate data gathered in metabolite separation, detection, identification and quantification through a series of latest technological results. High-performance (HPLC) or ultra-performance (UPLC) types of liquid chromatography (LC), gas chromatography (GC) and capillary electrophoresis (CE) are the most common separation methods. Mass spectrometry (MS), nuclear magnetic resonance (NMR), near infrared spectrometry (NIR) and Fourier transform infrared spectroscopy (FTIR) are among the most widely applied detection methods. In Foodomics, the majority of separation analyses were implemented by combining different detection methods (Mozzi et al., 2013). High throughput

data analysis and bioinformatics are also important in metabolomics and the modern metabolomacist may come from either a scientific or an informatics background and must be able to comment and understand both.

The application of metabolic profiling of the hydrophilic components in cheese represents a major advance toward scientifically expressing its sensory characteristics (Ochi et al., 2013). Ochi et al., (2012) conducted metabolic profiling using GC-MS, targeting hydrophilic low-molecular weight components in hard/semi-hard Cheddar and Gouda cheeses with various degrees of ripening, successfully resulting in the construction of highly precise sensory prediction models for 2 sensory attributes expressing important parts of maturation, "rich flavour" and "sour flavour". Additionally, Ochi et al., (2012) reported using a metabolomics based cheese quality evaluation tool at manufacturing sites. Metabolic fingerprinting using gas chromatography-flame ionization detector successfully reconstructed the sensory prediction model. Similar reports showed that monitoring low-molecular hydrophilic compounds, was capable of predicting significant sensory features linked to maturation, identify compounds contribution in associated with hard cheeses (Ochi et al., 2013).

2.8.1 Nuclear Magnetic Resonance (NMR)

High-resolution Proton Nuclear Magnetic Resonance spectroscopy (^{1}H NMR) is extremely helpful in food chemical analysis, potentially providing an overview of a broad variety of compounds in the food matrix in a single experiment (Mannina et al., 2012),. In conjunction with multivariate chemometric techniques, ^{1}H NMR is a powerful instrument for metabolic fingerprinting food products and highlighting the differences in metabolic profiles associated with standard processing and storage requirements (Savorani et al., 2010). This strategy opens the option of applying NMR spectral information for independent extensive food chemical processes controlling, like in Process Analytical Technology (PAT) (van den Berg et al., 2013).

Interestingly, only a few studies have been carried out on cheeses among the ^{1}H NMR-based metabolomic applications in food science (Brescia et al., 2005; Consonni and Cagliani, 2008; Gianferri et al., 2007; Lamanna et al., 2008; Mazzei and Piccolo, 2012; Piras et al., 2013; Rodrigues et al., 2011; Shintu and Caldarelli, 2006). The main reason for this maybe the

complicated nature of flavour development with extensive knowledge gaps in microflora interactions, cheese composition, flavour and maturation (Piras et al., 2013).

2.8.2 Principles of NMR

NMR exploits an intrinsic property of nuclei called spin, which is described by the spin quantum number, *I,* and can take half integer values. Nuclei with $I = ½$ are most commonly used for NMR since those with $I = 0$ are not visible by NMR and those with higher values of I are more difficult to observe. ^{1}H is the most commonly used nucleus for NMR due to its high natural abundance, high presence organic molecules and large gyromagnetic ratio, which makes it sensitive in comparison to other nuclei.

If spin ½ nuclei, such as protons, are placed in an external magnetic field they can be thought of as adopting one of two orientations, with their dipole aligned with or against the magnetic field. This generates two energy levels which can be populated by the nuclei. Slightly more nuclei occupy the lower energy level leading to a bulk magnetisation of the sample which aligns with the applied magnetic field and can be modelled as a vector pointing in the z direction.

The equilibrium or the system can be perturbed by applying a radio frequency pulse perpendicular to the applied magnetic field causing the bulk magnetisation vector to tilt into x-y plane and precess about the direction of the external field (the z axis). The frequency of the precession is equal to the difference in energy between the two levels and is known and the Larmor frequency. As the system returns to equilibrium, the bulk magnetisation vector moves back to align with the z axis. The precession of the magnetisation vector in the x-y plane, and its decay as the system returns to equilibrium, is recorded in the time domain as the free induction decay (FID).

In order to improve the signal to noise ratio, this process or perturbing the bulk magnetisation and then recording the FID is repeated a number of times and the FIDs summed. The frequency domain spectrum is then generated by applying a Fourier transform to the FID (Figure 2.5). Protons in different chemical environments give rise to peaks at different frequencies because their energy levels, and hence the Larmor frequency, vary slightly depending on the environment of the proton. Interactions, known as couplings, between protons in neighbouring

groups can cause peaks to split into two or more peaks generating multiplets which give further information on the chemical structure of the molecules concerned.

The positions of peaks in a NMR spectrum are measured on the chemical shift scale. Chemical shift, δ, is defined by the frequency separation of a peak from standard, which has been assigned a chemical shift of zero. It is measured in parts per million (ppm) and is given by the formula:

$$\text{Chemical shift, } \delta(\text{ppm}) = 10^6 \text{ x } \frac{\text{frequency} - \text{frequency of reference}}{\text{frequency of reference}}$$

As the differences in frequencies are very small, they can be measured, and scaled if needed.

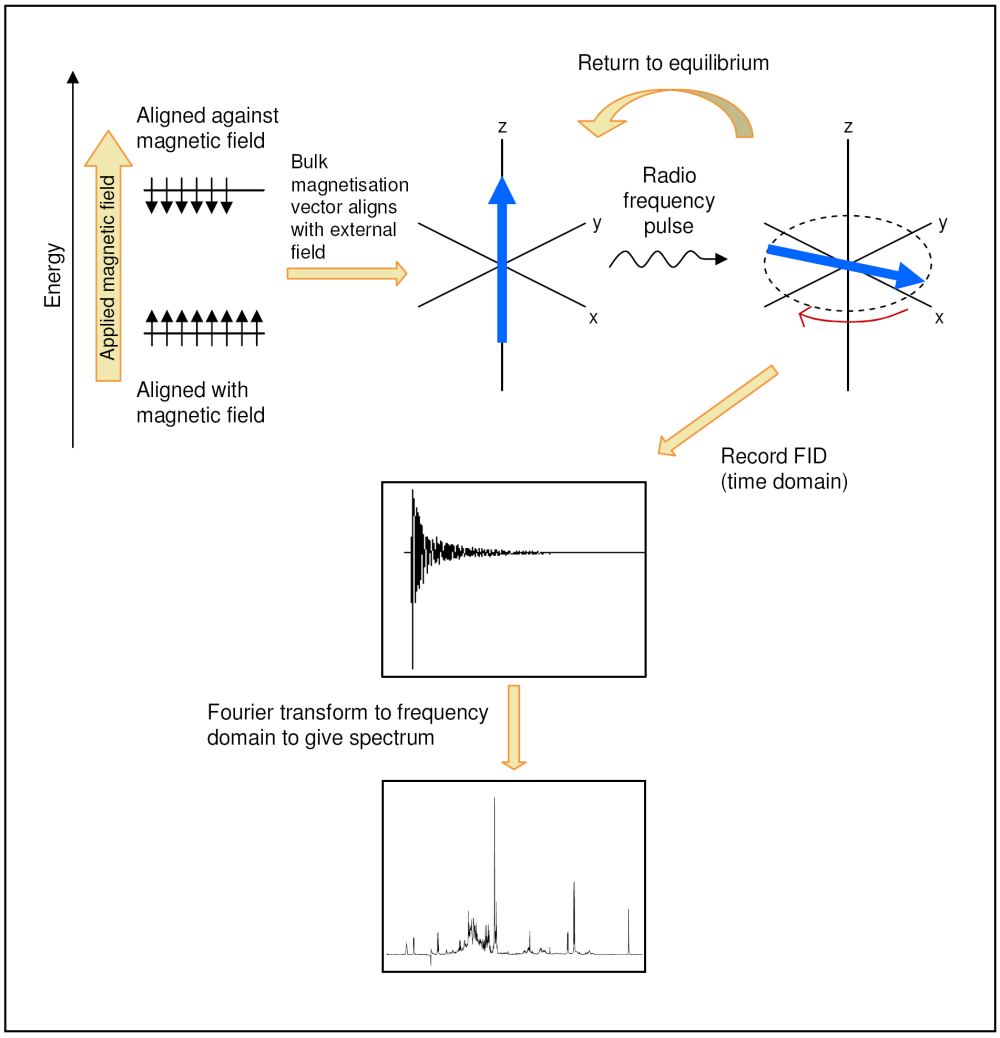


Figure 2-5 Summary of the principles of NMR

NMR is more than just Fourier transformation. Low-resolution NMR relaxometry deals with the quantitative determination of the most abundant compounds in a sample by assessing the NMR signal decline, rates and amplitudes. The most important advantages of low-resolution NMR is that it does not require any pre-treatment of the sample to be investigated and it has the ability to give information on physical structures of a material, such as in stretched curd cheese (Angelis et al., 2000). In addition, once established, the standard protocols can be readily transmitted to on-line quality control applications on the fast evaluations acquired by low-resolution NMR instruments.

2.8.3 Solid Phase Micro Extraction

The use of solid phase micro extraction (SPME) dates back to 1992 when it was developed for extracting volatile compounds from waste materials (Arthur et al., 1993). This method is based on the adsorption of volatile compounds onto the binding site(s) of a coated fibre. This fibre is then inserted into a GC inlet and those adsorbed compounds are thermally desorbed into the GC analytical column. Unlike other extraction methods, SPME uses no solvent. This property makes SPME very distinct and saves time and labour for sample preparation. Several types of SPME fiber have been developed using different coating materials such as polyacrylate (PA), polydimethylsiloxane (PDMS) and carboxen (CAR).

There is no doubt that the coating material may be selective toward some groups of compounds. For example Chin et al. (1996) showed that polyacrylate coated fiber was more effective than the polydimethylsiloxane nonpolar fiber for the analysis of short chain fatty acids. Volatile compounds adsorbed on the polyacrylate fiber were 3 to 20 times more than that with a nonpolar fibre in terms of chromatogram peak areas. SPME has been used in many research areas including pharmaceuticals, drug testing, pesticide analysis and many more.

In comparison to commonly extraction techniques such as solvent extraction, distillation and purge-and-trap, SPME is solvent free, rapid, simple, low cost and with good repeatability that renders it suitable for flavour analysis. The precision of SPME sampling is typically ~5% RSD and can be as low as 1 % RSD with an autosampler (Zhang and Pawliszyn, 1993). As one may suspect, SPME does exhibit some selectivity. Yang and Peppard (1994) compared three sampling methods: direct injection, SPME liquid sampling and SPME headspace sampling and indicated that SPME headspace sampling exhibited good sensitivity at 0.01 to 1 ppm

concentration toward ethyl acetate, hexanoic acid, y-hexalactone, limonene, and phenylethyl alcohol using a polydimethylsiloxane fibre. However, no detection was observed of methylbutyric acid and several other compounds. In a further study, two polyacrylate and polydimethylsiloxane based SPME fibres were examined by Yang and Peppard (1995). With headspace sampling, the polyacrylate fibre resulted in more chromatographic peaks with larger areas than the polydimethylsiloxane fibre. In the same study, 31 flavour compounds with approximately equal masses were prepared and examined using these two fibres. The polyacrylate fibres did not preferentially adsorb less-volatile compounds but showed some preference toward higher polarity compounds. The authors suggested that both coatings are excellent for analysis of food flavour compounds.

It is commonly known that heating and agitation help the release of analytes into headspace that facilitates headspace sampling. The adsorption mechanism of SPME fiber has been studied extensively by Zhang and Pawliszyn (1993). Rapid binding of volatiles onto coated fiber was observed on a static headspace with an agitated aqueous phase, much faster than that from a static aqueous phase. As explained by Zhang and Pawliszyn (1993), the rate of SPME headspace extraction is affected by rate of mass transfer of analyte to the surface of sample matrix, rate of desorption of analytes from the sample surface, convective transport of the analytes to the extraction fiber as well as rate of adsorption for analyte onto the fiber coating. Proper agitation and increasing the extraction temperature facilitate the mass diffusion.

As mentioned by Yang and Peppard (1995), the SPME adsorption is a single batch mechanism with theoretical plates N <= 1 suggesting that SPME has generality to volatile compounds. There are three phases involved in SPME extraction. Equating the observed GC peak area or the amount of analytes back to its concentration in the sample matrix needs to be established. One of the problems associated with SPME headspace sampling is the quantitation of analytes in the sample matrix.

SPME and multivariate statistical analysis was performed to monitor ripening changes in cheese volatile compounds caused by maturation by Milosavljević et al. (2012). Two different types of Pasta-Filata cheese were evaluated for their volatile profiles and ripening-induced changes at four different phases of ripening (1, 5, 20, and 30 days). They showed that the class distribution of volatile compounds provides useful data about the type of cheese and the

ripening stage. When the relative ratio of the constituent classes was in question, the extraction with a polyacrylate (PA) fiber gave a more realistic cheese volatile profile, while the PDMS fiber was easier to recognize the contribution and concentration of single dominant volatile compounds.

2.8.4 Gas Chromatography-Mass Spectroscopy (GC-MS)

Gas chromatography (GC) is usually used to separate volatile compounds, or compounds that can be made volatile. Mass spectrometry may be used to identify compounds after or during gas chromatography separation and tentative identifications may be made using gas chromatography/olfactometry (GC/O) analysis retention indices and flavour profiles.

A mass spectrometer is generally made up of an ion source, a mass-selective analyser, and an ion detector. Atoms or molecules are ionized and specific mass and charge fragments are created. Based on the mass-to-charge ratio (m/z), the mass-selective analyser then divides the ions. Molecules have unique fragmentation patterns that can provide structural data, chemical formula and molecular weight of the molecule (Ravindranath, 1989). While traditional gas chromatography analyses are a strong and sensitive instrument for identifying and quantifying volatile compounds in food, they cannot provide data on the overall contribution of individual components to the flavour and aroma. Basically, only a tiny proportion of all compounds are aroma-active and making a significant contribution to a specific aroma and flavour. furthermore, the total quantity of a compound is not directly associated with its flavour effect, since the concentration can be below the sensory threshold of humans (McGorrin, 2002).

2.9 Scope of the study

Currently, the characteristics of soft cheese are determined using sensory panels, which are time-consuming, and laborious. This project will first develop and test new analytical methods as part of a metabolomics-based approach to study will investigate and characterise the ripening process of specific soft cheeses. Metabolomics is likely to be useful for the study of cheese ripening as it specifically looks at patterns in biological profiles. Many small molecules contributing to a flavour profile in cheeses; the highly interconnected nature of the taste also means that important information is most likely to be found in correlation patterns as opposed to individual signals. By measuring the changes that occur in both primary and secondary metabolites during cheese ripening process and their contribution to cheese flavour in detail, a

much richer picture of the overall flavour of a product can be obtained. I will also be able to test for the bio (or taste) markers of the process going wrong. Detecting such processes earlier than is possible at present will enable corrective action to take place, thus saving both time and money.

The primary techniques that will be used in this work are including Nuclear Magnetic Resonance spectroscopy, Near Infrared Spectroscopy (NIR), Gas Chromatography tandem Mass Spectroscopy (GC-MS) and Solid phase micro-extraction (SPME) – as outlined above. Data from all of these methods will be used to assess biochemical profiles of selected soft cheeses during the ripening process. The use of such methods shows great promise as tools for the rapid analysis of cheese characteristics, which will save time, labour and operational costs for the industry and cheese research. Once data on the changes that occur over time have been obtained it will be possible to use this information to improve soft cheese processing and manufacture on a large scale.

Chapter 3 GAS CHROMATOGRAPHY MASS SPECTROSCOPY (GCMS)/SOLID PHASE MICRO EXTRACTION (SPME)

3.1 Introduction

For the concentration and analysis of volatile organic compounds, solid phase microextraction technique (SPME) was used. This technique is fairly simple and similar to dynamic purge and trap analysis. It demonstrates good reproducibility, linearity and precision (Taylor and Linforth, 2003; Taylor and Linforth, 2009).

Solid phase microextraction technique only requires small quantities and isolate volatile and semi volatile compounds from samples. It is a moderate method that enables the natural contents and sample composition to be preserved. Issues can be observed in isolating less volatile compounds or with high matrix affinity, such as isolating polar compounds from polar nature matrices. In these situations, derivatisation is used or the conditions of extraction, like fibre type or temperature, must be calibrated to optimise recovery and sensitivity as discussed previously in chapter two (Milosavljević et al., 2012).

3.2 Material and methods

3.2.1 Manufacturing and sampling of camembert cheese

There are some key steps in cheese making and the finished product's flavour, aroma, and texture is defined by moisture content, salt level, pH, and microflora, and by monitoring and controlling the biochemical changes that happen during maturation process.

The production of surface-mould cheese starts with the selection of high-quality milk, both chemical and microbiological. Raw milk is still often used for commercial and farm cheese production; although, cheese milk is now normally pasteurized or heat treated immediately prior to us in order to kill pathogens, pasteurization eliminates some indigenous microorganisms and thus it is possible to produce cheese of a more standardised quality. The surface microflora is more complex than the centre, especially in traditional technology cheeses made from raw milk. The acidification process is started once the milk has been selected and

pre-treated (if required) and continues for up to 24 hours. Use a starter culture of known lactic acid producing bacteria is now almost universal practice, with the primary mechanism of producing acid at the acceptable rate during cheese production. The Camembert cheeses lactic starter bacteria are commonly homofermentative mesophiles (e.g., *Lactococcus lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris*) producing lactic acid through the hexose diphosphate mechanism. Due to the amount of starter added to cheese milk, the acidification rate for Camembert cheese is slower than for hard cheeses such as Cheddar, (Sousa, 2003). At the same time, it is possible to inoculate the milk with microorganisms that later form the surface flora (e.g suspension of *P. camemberti* spores).

To promote coagulation, generally $CaCl_2$ (0.02 percent) is added to the milk. Coagulation of the casein component of the milk protein system includes gel formation that interferes with salts, fat, water, and also lactose. Chymosin is the main proteinase used for cheese production in traditional rennet and its major role is to primarily hydrolyse the micelle stabilizing protein Phe_{105}–Met_{106} bond of, k-casein, which results in coagulation in the presence of Ca^{2+} at temperatures higher than 20 ^{0}C.

A gel or coagulum induced by rennet is almost stable until it is cut or broken, resulting in syneresis (the contraction of a gel accompanied by the separating out of liquid). Camembert cheese has a high moisture content (45 to 55 percent) that prevents excessive syneresis, the coagulum is cut or broken into very large pieces, and the curds are not cooked and only stirred slightly in the whey. Typically, an alternative method of whey and curd separation is performed, called “dipping” where the uncut coagulum is scooped from the container into perforated moulds, where drainage occurs. To enable manufacturing on an industrial scale, Camembert coagulum is first cut into large cubes and then transferred to moulds.

The body of curds in the mold is turned over a period of 3 hours at intervals after molding and allowed for cooling overnight; Camembert is pressed only under their own weight. The pH of camembert cheese is low shortly after production (4.6 to 4.9). Camembert cheeses are usually salted for long enough by immersion in the brine to give a total salt content of 1.5 to 2.5 percent. Salting generally stops acid production in the curd, therefore, it preventing more pH decrease. In addition to having a direct effect on cheese flavour, the salt promotes curd syneresis and perhaps affects moisture content by reducing curd water activity and influencing rennet

activity, also indigenous milk enzymes, and other enzymes. The salt Na^+ exchanges with curd calcium matrix, resulting in protein dissociation, and further enhances the protein matrix emulsification of fat. During cheese making process mould spores are introduced to Camembert chesses as a secondary starter. *P. camemberti* spores are usually sprayed on the surface of each freshly shaped cheese, although it is now industrial practice to inoculate cheese milk with spores before clotting (Sousa, 2003).

These spores appear in a white mould growing on the outside of each cheese under optimum conditions (10 to 12 ^{0}C and 85 to 95 percent relative humidity (RH) for 10 to 12 days). This mould gives the cheese specific characteristic appearance and produces enzymes during maturation that play an important role in lipid and protein hydrolysis. Following the development of the mould, cheeses are packaged in waxed paper and placed in boxes of wood or cardboard for another 7 to 10 days of maturation at 4 ^{0}C. In traditional Camembert cheeses, where maturation continues for up to 12 weeks, the mould is subsequently overgrown with *Brevibacterium linens* and other coryneform bacteria to form the yellow crust, a distinct sulphury aroma and also the very soft texture of extra-mature Camembert (Sousa, 2003).

The manufacture process flow for the production of Camembert cheese as used in this project is shown below in Figure 3.1.

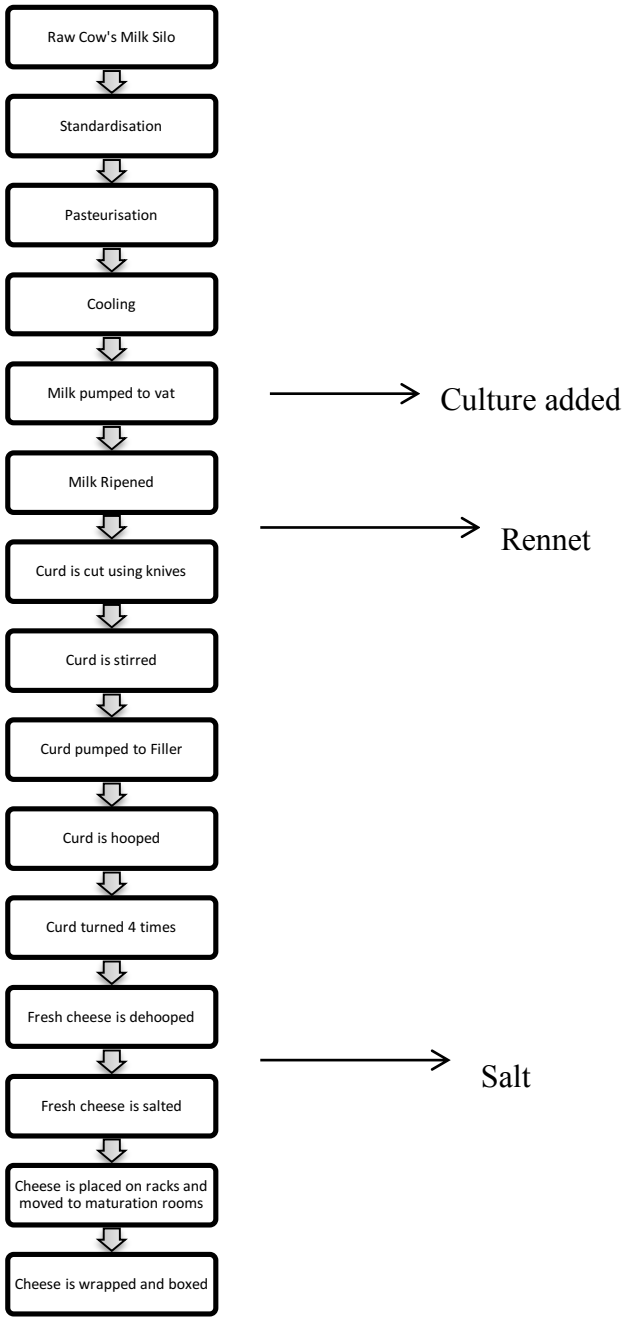


Figure 3-1 Camembert process flow

Methods

Curd and Camembert cheese (Tasmanian Heritage) were sourced from LD&D Foods Pty Ltd, as wrapped units immediately after demoulding. They were packed in carton cases (5 units per case) (see Table 3.1 for nutrition and label information). These cheeses were ripened at the fridge (2-4℃) for 30 days. GC-MS/SPME was undertaken at day 1, 5, 10, 15, 20, 25 and 30. All the analyses were performed in duplicate.

<table>
<tr><th colspan="3">Nutrition Information</th><th>Label Information</th></tr>
<tr><td colspan="3">Serving per package:5
Serving Size 25 g</td><td rowspan="10">Name: Mini Camembert
Name and address of supplier: LD&D FoodsPty Ltd, 145 Old SurreyRoad, Burnie, Tasmania 7320.
Storage Condition: Refrigerate at or below 5℃
Net Weight: 125g
Best Before: Use by DD.MM. YY
Use Instructions: Best eaten at room temperature</td></tr>
<tr><td>Nutrient</td><td>Per Serve</td><td>Per 100g</td></tr>
<tr><td>Energy</td><td>317 kJ</td><td>1270kJ</td></tr>
<tr><td>Protein</td><td>4.8g</td><td>19g</td></tr>
<tr><td>Fat- Total</td><td>6.3g</td><td>25g</td></tr>
<tr><td>-Saturated</td><td>4g</td><td>16g</td></tr>
<tr><td>Carbohydrate- Total</td><td><1g</td><td><1g</td></tr>
<tr><td>-Sugars</td><td><1g</td><td><1g</td></tr>
<tr><td>Sodium</td><td>171mg</td><td>685mg</td></tr>
<tr><td colspan="3">Ingredients: Pasteurised Milk, Salt, Mineral salt (509), Culture, Rennet.</td></tr>
</table>

Table 3-1 Tasmanian Heritage cheese nutrition and label information

3.2.2 Extraction of volatile compounds

Frozen Camembert was crushed under liquid N_2 using a pestle and mortar and then 5 ±0.5 g of samples were taken and placed in 20 mL vials, sealed with an aluminium cap provided with silicone septum. The sample vial was placed for 5 min in a 45 ℃ heating block to equilibrate and the septum was then pierced with SPME needle. The fibre was exposed to the headspace of the sample for 30 minutes. The fiber was then retracted into the needle after the extraction time and transferred to the injection port immediately and desorbed in a splitless mode at 250 °C for 5 minutes. Divinylbenzene/ Carboxen/ Polydimethylsiloxane (DVB/CAR/PDMS) fiber was used for extraction of volatiles, as the most effective fiber based on previously published data (Majcher et al., 2011).

3.2.3 GC-MS procedure

Samples were desorbed directly (without delay) by manual injection into the GC injection port after extraction. A Hewlett Packard 5973 Mass Selective Detector (MSD) GC-MS was used for GC-MS analysis of the cheese fat extracts. A nonpolar capillary column (DB-5ms, 30 m length, 250 µm i.d., 0.25 µm df, Agilent J&W Scientific) was used. Helium was used as as the carrier gas at a flow rate of 1.6 mL/min. The oven temperature was set at a rate of 6 °C/min from 40 °C to 190 °C with an initial holding time of 1 min. The following MS conditions were: inlet temperature 250 °C Transfer line and capillary direct interface temperature 280 °C; ionizing energy 70 eV; mass range 41 to 415 a.m.u; EM voltage (+47 V); scanning rate 3.8 scans/s. In the splitless mode, each extract was injected and on each sample duplicate analyses was undertaken.

The relative abundance (percent peak area) of two independent replicate samples of each group compound identified by GC-MS is given in Figure 3.2.

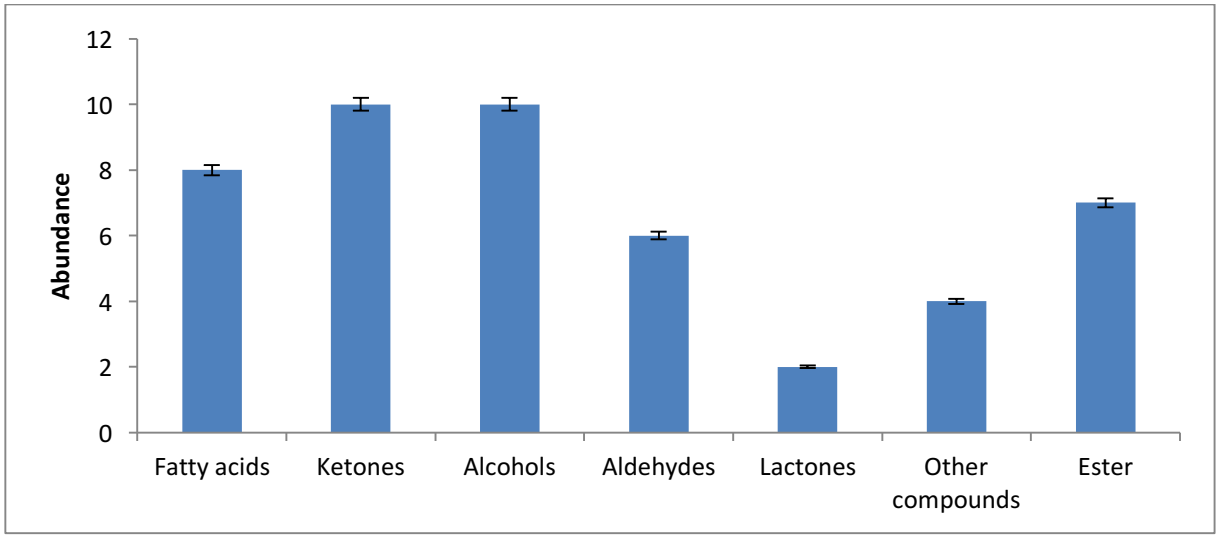


Figure 3-2 Relative abundance of different compound groups

3.3 Statistical analysis

All data were loaded into Microsoft Excel (2007) spread sheet and statistical analysis performed using principle component analysis (PCA) using data obtained from cheese samples of different maturity classes. The correlation between data sets was carried out using partial least squares regression (PLSR).

3.4 Result and discussion

3.4.1 Head space analysis by GC-MS

Selection of a suitable SPME fiber is usually applied based on the target analytes' volatility and polarity. DVB/CAR/PDMS fiber has a strong capacity for extraction of the small volatile compounds. It was therefore chosen as the best option with high sensitivity for small volatile molecules, mainly sulphur compounds in cheese (Lecanu et al., 2002). Several previous studies have reported superior extraction efficiency for DVB/CAR/PDMS which is in agreement with the results obtained here. For instance, Boltar et al. (2015) evaluated the formation of volatile compounds in different seasons during ripening of Nanos cheese. Their analysis was performed by solid-phase micro extraction gas chromatography–mass spectrometry using DVB/CAR/PDMS. They confirmed this fiber shows better response to volatile compounds rather than CAR/PDMS.

Temperature is crucial for greater efficiency of HS-SPME extractions as it affects the diffusion rate and partial pressure of analytes into the headspace (Câmara et al., 2007). In this study, the amounts of volatile compounds of 30 days ripened Camembert cheese extracted with SPME, were compared at different temperatures (25, 35, 45 and 50^{0}C) and time (see Figure 3.3 and Table 3.2). In this study analysing of SPME on curd, day 1, 5, 10, 15, 20, 25 and 30 days ripened Camemberts cheese was performed for 10, 20 and 30 minutes, respectively, the aim being to identify the ideal parameters for the experiments. These data are summarised in table 3.2 and figure 3.3 respectivly.

Temperature (^{0}C)	Time (minutes)		
	10 min	20 min	30 min
25^{0}C	43	39	104
35^{0}C	54	40	161
45^{0}C	57	142	214
50^{0}C	62	180	216

Table 3-2 Abundance of compounds extracted by HS-SPME in 30 days ripened Camembert cheese at different time and temperature

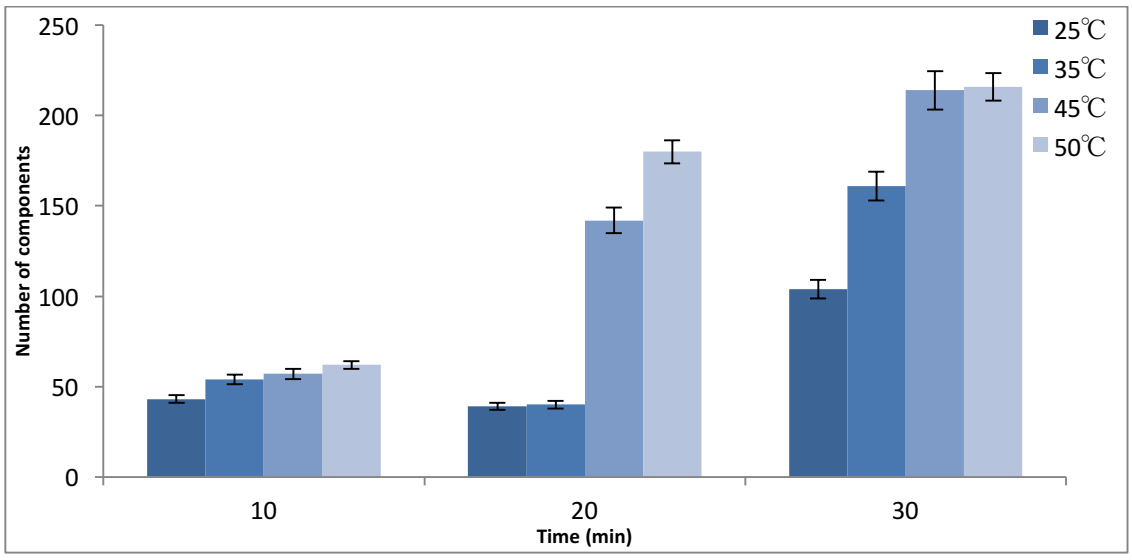


Figure 3-3 Extraction of the aroma compounds in 30 days ripened Camembert cheese

High temperatures usually increase the process of mass transfer and enable analytes dispersion from food matrix to the headspace. High temperatures, can also have a negative impact on the

adsorption of analytes on SPME fiber due to reduced partition coefficients between headspace and fiber (Zhang and Pawliszyn, 1993). On the other hand, higher temperature may lead to produce rancid compounds which can change the final flavour of the cheese. An increase in producing rancid compounds was observed with increasing extraction temperature (see Figure 3.4). This meant it was important to balance the extraction times and temperatures used for the study.

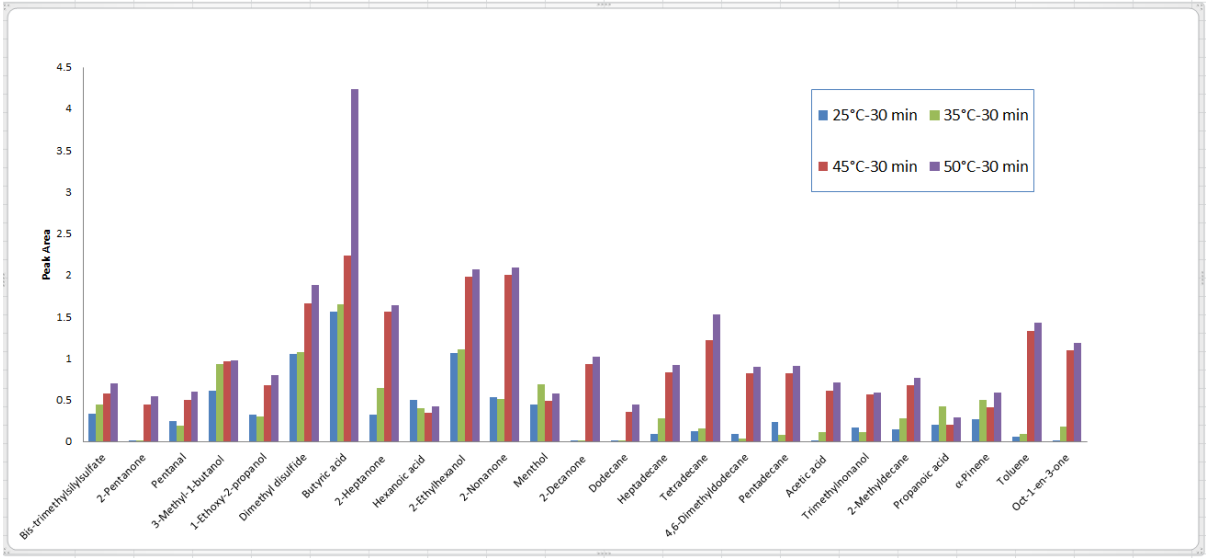


Figure 3-4 Extraction of the aroma compounds of Camembert cheese in 30 minutes

Butanoic (butyric) acid, which plays an important role in the flavour of many cheese types, including Camembert has a rancid cheese-like odour. However, large quantities of this volatile compound are unwanted, and may form from fermentation of butyric acid (Curioni and Bosset, 2002). Here it was found that a temperature of 45^0C was favoured because the very high amount of Butyric acid with unpleasant odour observed at 50°C (see Figure 3.5).

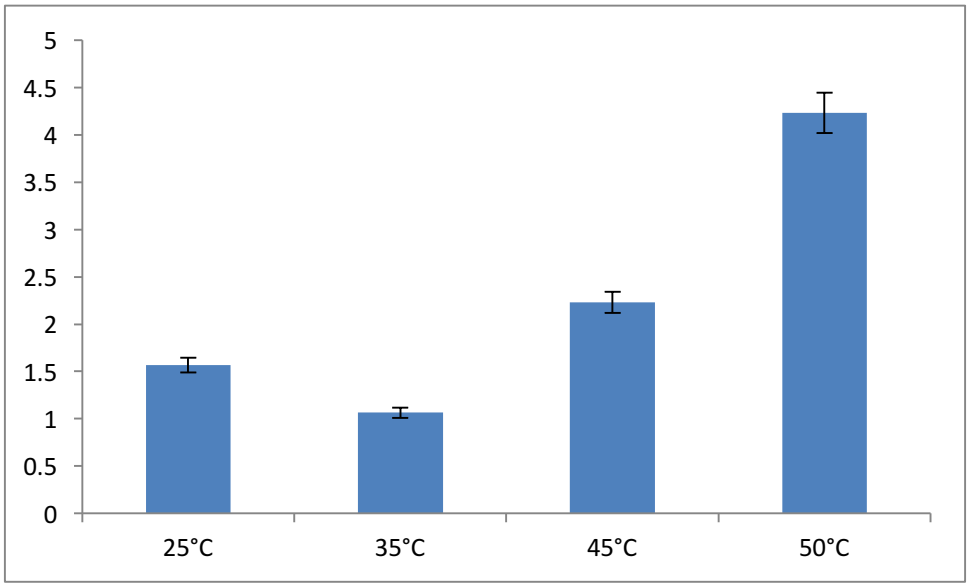
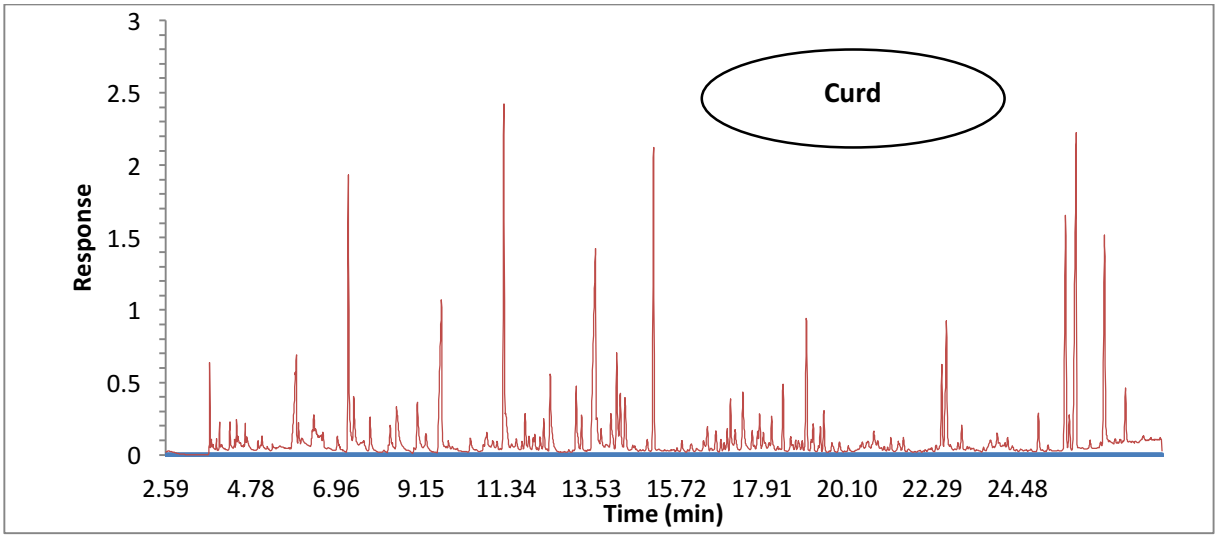
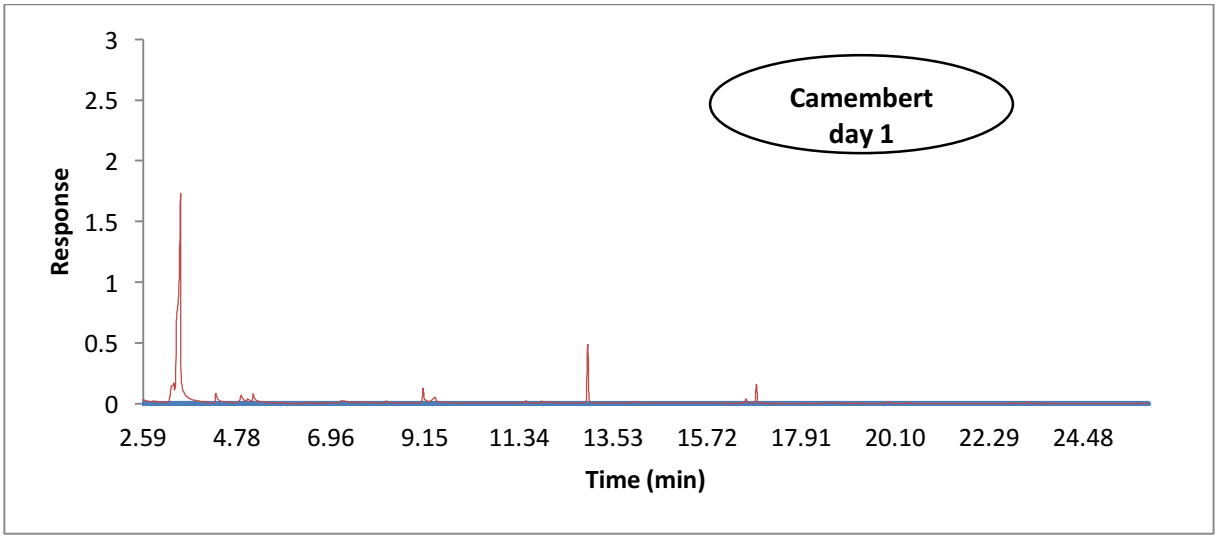


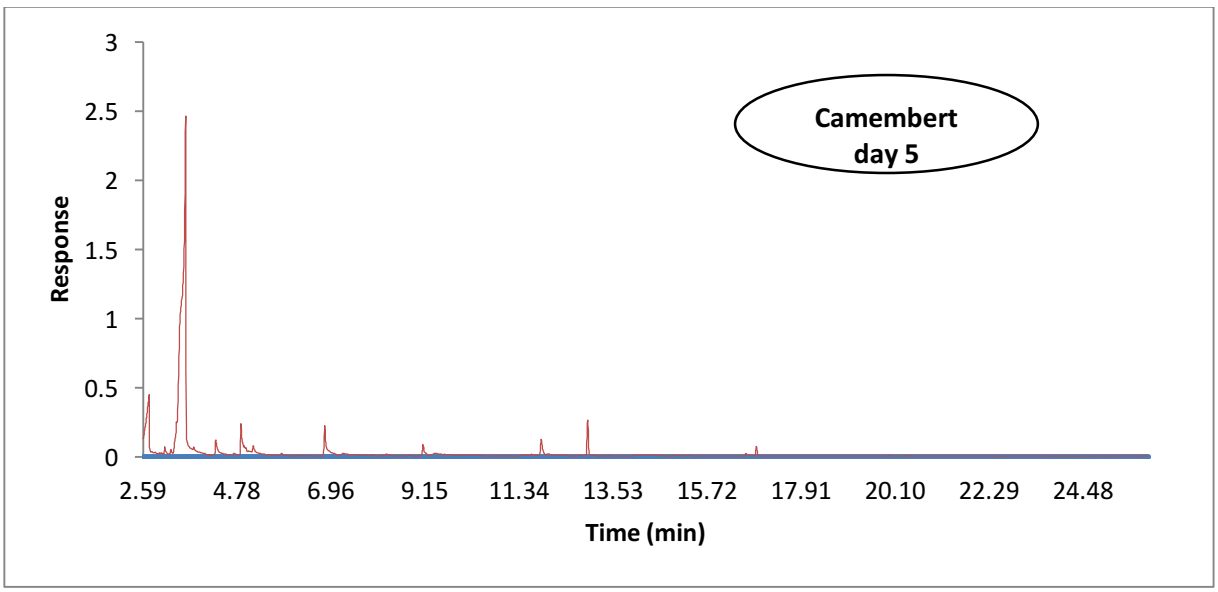
Figure 3-5 Changes in Butyric acid in Camembert cheese at differing SPME extraction temperatures

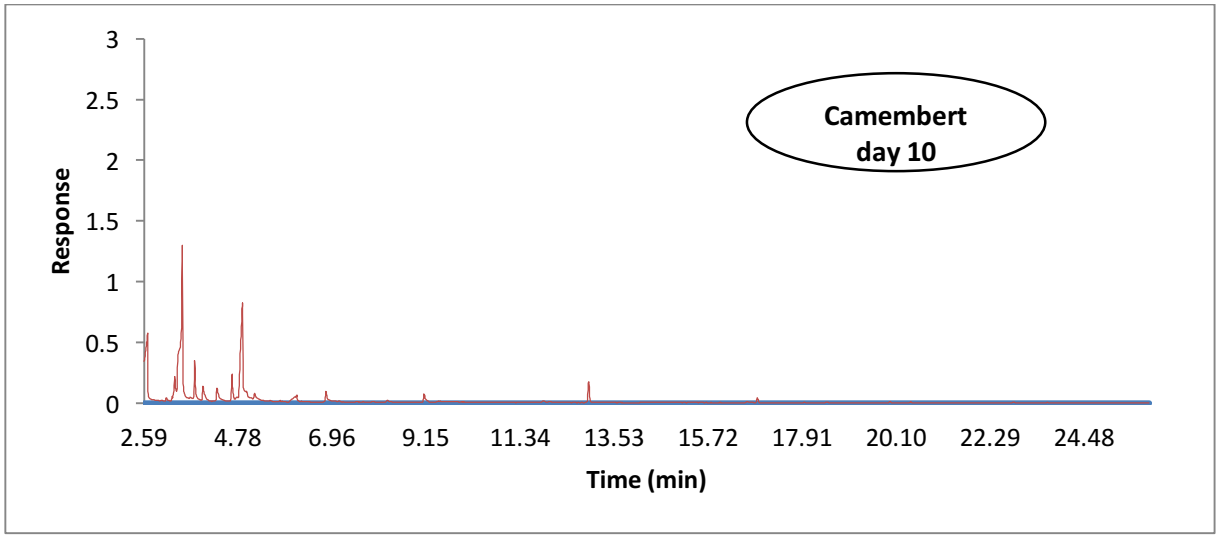
After evaluation of the main parameters affecting the efficiency of SPME extraction in Camembert cheese, the following sampling conditions were selected as optimum within the range tested: DVB/CAR/PDMS fiber; 45^0C extraction temperature; 30 min extraction time. Over a period of 30 minutes at 45^0C, 214 compounds were detected in 30 days ripened Camembert cheese.

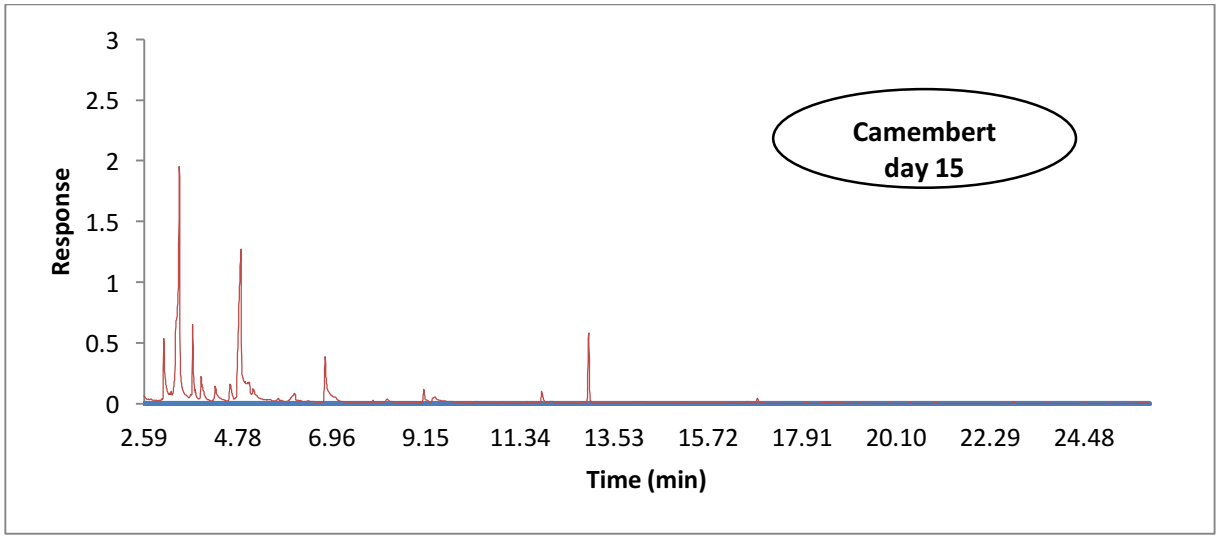
Comparing the optimized method (50/30 µm DVB/CAR/PDMS fiber, 45ºC extraction temperature, 30 min extraction time) with a published method (Greene et al., 2008), for all compounds tested, a significant increase in response was observed. This meant I was able to pick up more compounds than a published method and so I was satisfied that the procedure was viable and useful. I was then able to use the method to look at how flavour compounds developed in the Camembert over time. The results of this study are show in figure 3.6 below. Please note that this figure has multiple panels and that the curd sample is raw, before the pasteurisation occurred.

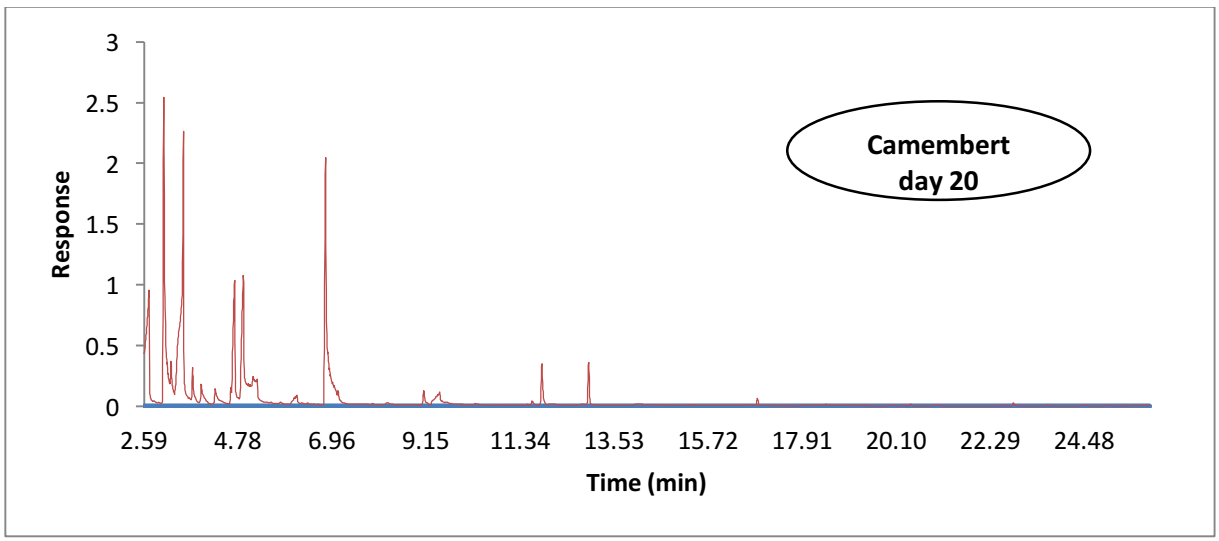

Curd
Response
Time (min)
3
2.5
2
1.5
1
0.5
0
2.59
4.78
6.96
9.15
11.34
13.53
15.72
17.91
20.10
22.29
24.48


Camembert
day 1
Response
Time (min)
3
2.5
2
1.5
1
0.5
0
2.59
4.78
6.96
9.15
11.34
13.53
15.72
17.91
20.10
22.29
24.48


Camembert
day 5
Response
Time (min)
3
2.5
2
1.5
1
0.5
0
2.59
4.78
6.96
9.15
11.34
13.53
15.72
17.91
20.10
22.29
24.48


Camembert
day 10
Response
Time (min)
3
2.5
2
1.5
1
0.5
0
2.59
4.78
6.96
9.15
11.34
13.53
15.72
17.91
20.10
22.29
24.48


Camembert
day 15
Response
Time (min)
3
2.5
2
1.5
1
0.5
0
2.59
4.78
6.96
9.15
11.34
13.53
15.72
17.91
20.10
22.29
24.48


Camembert
day 20
Response
Time (min)
3
2.5
2
1.5
1
0.5
0
2.59
4.78
6.96
9.15
11.34
13.53
15.72
17.91
20.10
22.29
24.48

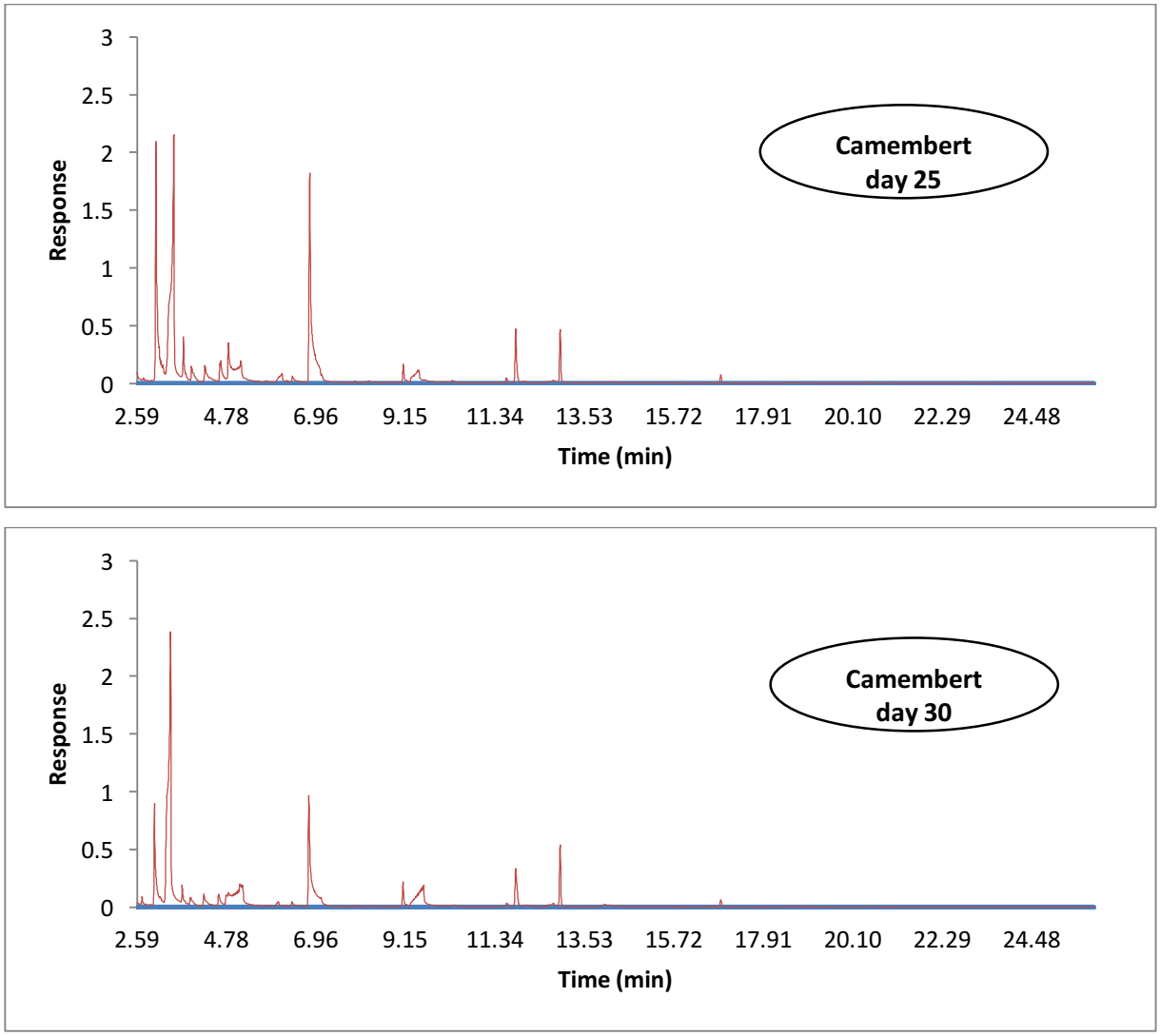


Figure 3-6 Comparison of GC-MS ``profiles of HS-SPME at different time of ripening in Camembert cheese

A total of 23, 9, 13, 13, 16, 20, 23, 32 volatile flavour compounds were detected and identified in the headspace of the curd on day 1, 5, 10, 15, 20, 25 and day 30, respectively. Many of these compounds were present in small amounts and so are not easily observed on the chromatograms in figure 3.6 due to differences in dynamic range with other compounds in the sample. The compounds were found to be mainly alcohols, ketones, esters and fatty acids. The characteristic flavour profile of Camembert was found to be roughly in accordance with previously published data in Camembert cheese (Kubíčková and Grosch, 1997; Lecanu et al., 2002; Pionnier et al., 2002) but more compounds were identified in my study.

3.4.1.1 General

A total of 47 compounds were detected including 8 acids, 7 esters, 10 ketones, 10 alcohols, 6 aldehydes, 2 lactones and 4 unknown compounds (See Table 3.3 and 3.4). Volatile compounds significantly changed during ripening, as most of the volatile compounds identified tended to increase and new compounds were formed during maturation, although a few compounds also decreased. A total of 23 volatile compounds isolated from curd and an important decrease of isolated compounds was found for Camembert Day 1. These specific characteristics during maturation could be correlated with the different metabolic mechanisms engaged in alcohols formation in cheese, which including lactose metabolism, methyl ketone reduction, metabolism of amino acids and linoleic and linolenic acids degradation (Molimard and Spinnler, 1996). The number of volatile compounds slightly increased during ripening for 30 days.

After 30 days of ripening, these results agree with the time when cheeses can be sold, so they developed most of the volatile compounds that form their characteristic profile at the time of purchase.

Aging time	Number of compounds
Curd	23
Camembert day 1	9
Camembert day 5	13
Camembert day 10	13
Camembert day 15	16
Camembert day 20	20
Camembert day 25	23
Camembert day 30	32

Table 3-3 Numbers of compounds detected at different time of ripening in Camembert cheese

Compounds	Abundance
Fatty acids	8
Ketones	10
Alcohols	10
Aldehydes	6
Lactones	2
Esters	7
Other	4

Table 3-4 Abundance of different chemical groups during ripening of Camembert

The evolution of volatile compounds grouped by their chemical nature is shown in Figure 3.7. Total carboxylic acid content, especially in the first 15 days, tended to increase during maturation. Lipolysis appeared to be the main linear carboxylic acids formation pathway with more than 4 carbon atoms. The shorter carboxylic acids, however, can also be derived by oxidation from ketones, esters and aldehydes (Molimard and Spinnler, 1996), like metabolic products of the metabolism of lactose, amino acids deamination and possibly lipid oxidation. During maturation, ketones gradually increased as very low levels of volatile ketones were detected at day 1 and increased during maturation. Volatile Esters followed a similar trend as explained for Ketones, but they tended to decrease after day 25. However, the levels of alcohols tended to decrease in the first days of ripening but then after day 10 they increased. This decrease is likely due to the increase of esters resulting from acids and alcohols esterification. The level of aromatic compounds at the beginning of the maturation tended to rise but then remained constant.

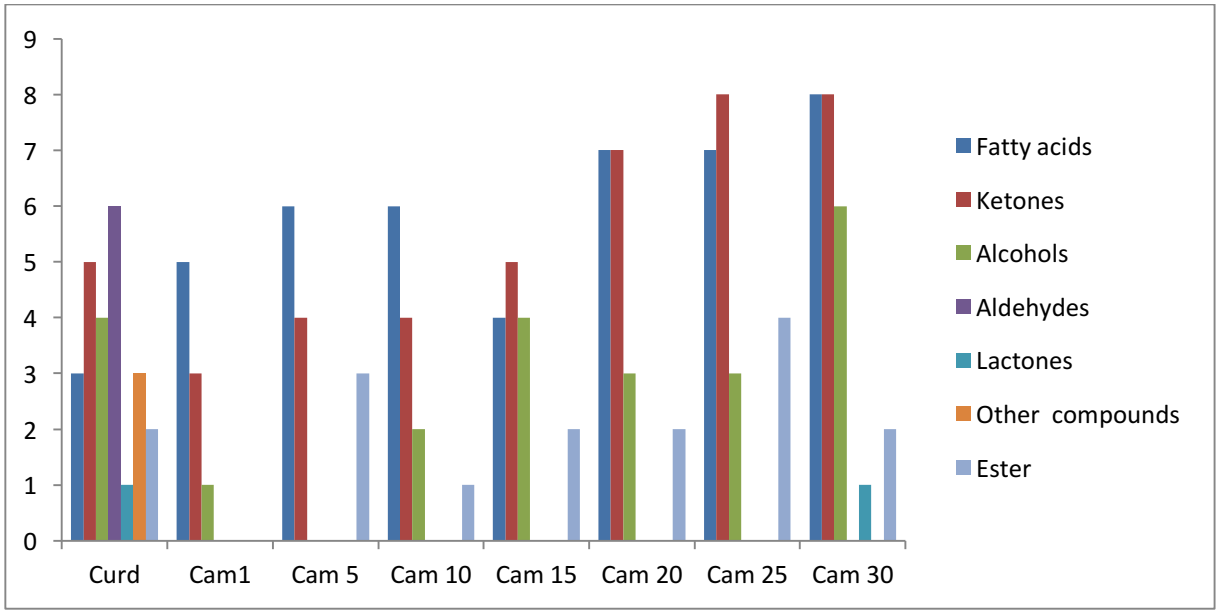


Figure 3-7 Changes of the main chemical groups of volatile compounds isolated in the Camembert cheese during ripening

3.4.1.2 Multivariate analysis

A principal component analysis (PCA) was performed on each day of maturation to determine the most important volatile compounds. PCA is a mathematical process for resolving data sets into orthogonal components, the linear combinations (principal components) of which approximate the original data to any desired degree of precision. Two principal components are sufficient in most cases to explain a large proportion of the variation in the original variables, resulting in considerable data compression. In this case, the principal components (PC) #1 and #2 respectively explained 46.9% and 22% of the total variability.

Figure 3.8 shows the loading plots of the different variables (coefficients of the eigenvectors) for the two first principal components (PC #1 and PC #2). Although all volatiles were included in the analysis, only volatile compounds which explained the variation of the data have been identified in the figure. Near the origin and on the negative axis of PC #1, are compounds related to the flavour of cheese at the beginning (day 1) to middle stage (day 15) of the ripening alcohols (3-methylbutanol, pentan-2-ol, 2-phenyl ethanol), carboxylic acids (hexanoic acid, acetic acid), aldehydes (benzaldehyde, octanal, decanal, dodecanal, nonanal, hexanal), ketones (acetoin), esters (ethyl formate, hexyl butanoate, ethyl acetate).

Regarding PC #2, on the positive axis, far from the origin and explaining an important part of the variation are located volatile compounds isolated to a higher extent in curd, such as carboxylic acids (propanoic acid, 2-methylpropanoic acid), esters (hexyl butanoate), ketones (acetophenone, acetone), alcohols (heptanol, ethanol, butanol), and lactones (butyrolactone). on the positive axis of PC #1, volatile compounds which are responsible for the unique flavour of ripened Camembert cheese (day 20 to 25) were located in the origin of the plane, such as, carboxylic acids (decanoic acid, butyric acid, isovaleric acid, hexanoic acid, octanoic acid, propanoic acid), esters (amyl acetate, butyl butanoate, ethyl butanoate), ketones (nonan-2-one, octan-2-one, hexan-2-one, undecan-2-one, 4-heptanone, acetoin), alcohols (nonan-2-ol, pentan-2-ol, propanol, 2-ethyl hexanal, 3-methyl butanol), and lactones (decalactone).

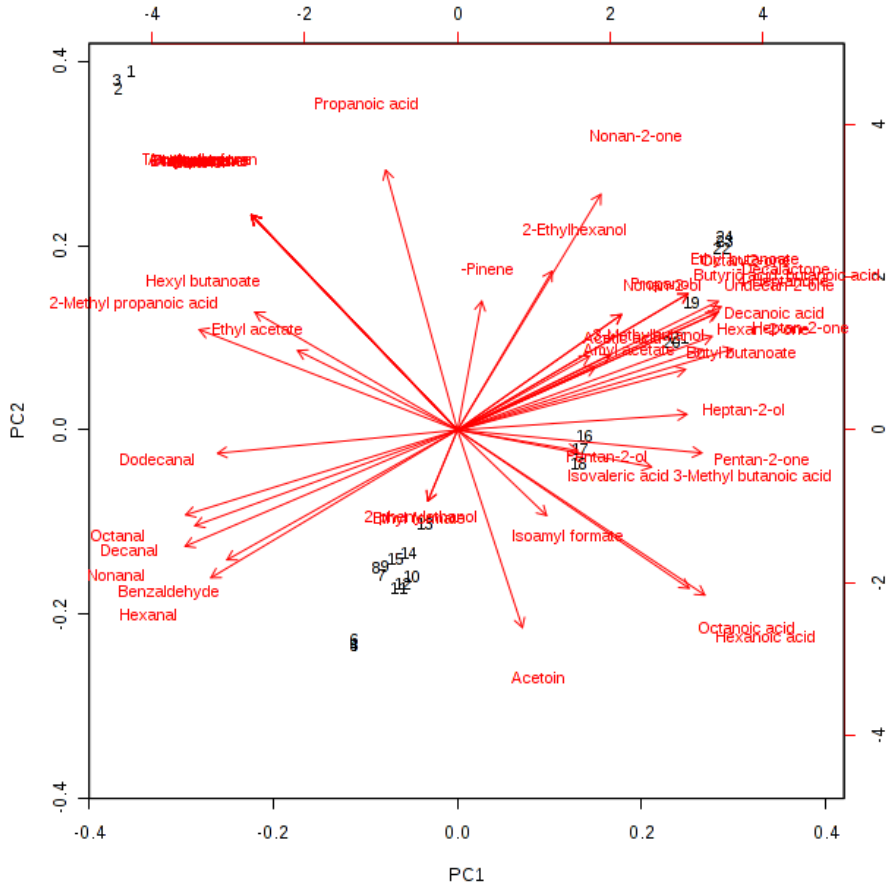


Figure 3-8 Loading plots after principal components analysis of the variables in the plane defined by the two first principal components (PC #1 and PC #2)

The distribution of the scores on the first two PCs (Figure 3.9) shows 3 separate groups of points, corresponding to the different days of ripening. Cheeses from 20 to 30 days of ripening are located in the positive area of PC #1 while in the negative area are those with early stage to middle stage of ripening (1 to 15). Curds are located in the positive area of PC #2, so PC #1 and PC #2 can explain differences between volatile compounds of each group. Such grouping confirmed the utility of GC-MS-SPME-PCA as a tool for the control of Camembert cheese ripening.

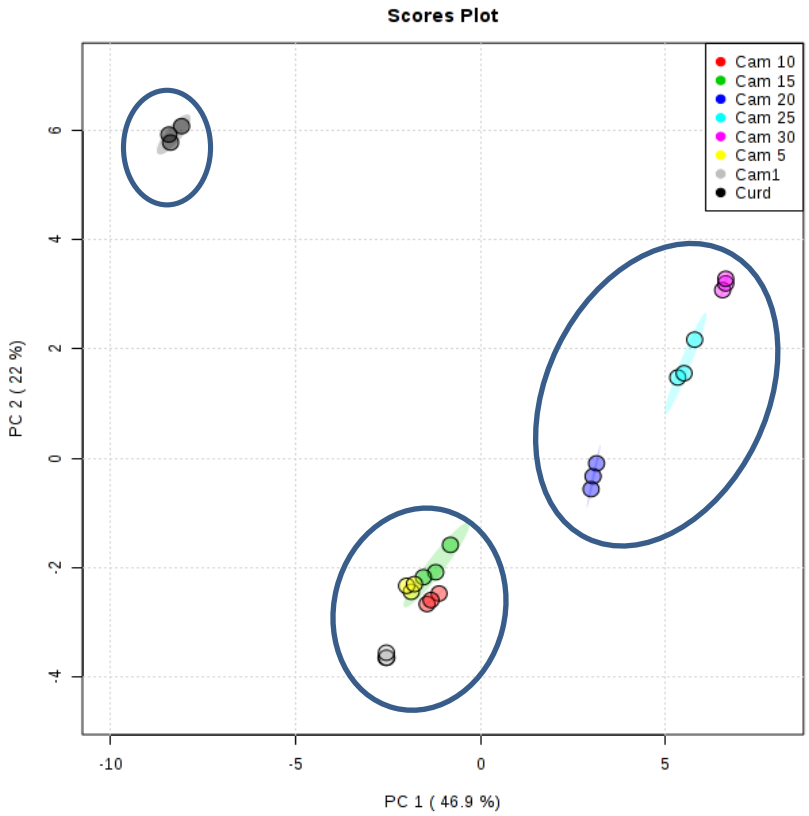


Figure 3-9 Scores plot after principal component analysis of the individuals in the plane defined by the two first principal components (PC #1 and PC #2)

Figure 3.10 shows the correct level of different volatile compounds on the heat-map. In addition, hierarchical clustering of the data showed that the cheeses on days 20 to 30 were very similar but could be distinguished by the clustering procedure.

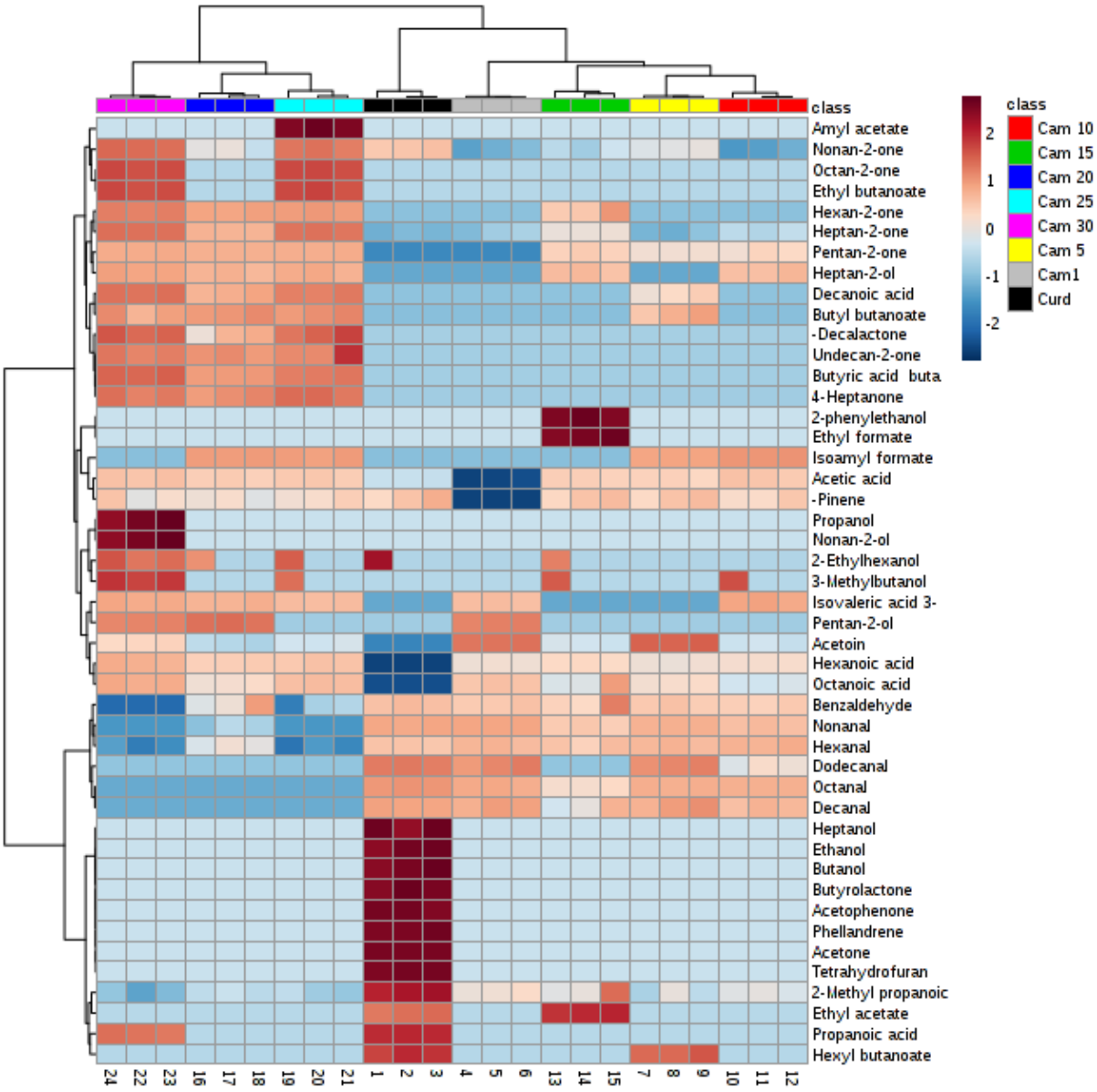


Figure 3-10 Hierarchical clustering of *volatile compounds* extraction during the ripening of Camembert cheeses. Extractions were measured at different ripening times and normalized the data. The log_{10} values of -fold changes in volatile compounds extraction are color-coded, with red indicating up-regulation and green down-regulation. The heat-map clusterings were constructed with Metaboanalyst

F. value, p. value and FDR of different volatile compounds isolated from Camembert cheese by SPME–GC–MS during 30 days of ripening are shown in Table 3.5.

Metabolites	f.value	p.value	FDR
Acetone	1388800	4.78E-45	2.20E-43
Tetrahydrofuran	17584	7.23E-30	1.22E-28
Isoamyl formate	17367	7.98E-30	1.22E-28
Propanoic acid	11409	2.30E-28	2.65E-27
Butyric	10893	3.33E-28	3.06E-27
Pentan-2-ol	10084	6.17E-28	4.73E-27
Acetophenone	9529.2	9.71E-28	5.68E-27
Octan-2-one	9509.4	9.87E-28	5.68E-27
Phellandrene	6440	2.23E-26	1.14E-25
Octanal	4916.1	1.93E-25	8.88E-25
Isovaleric acid	4328	5.35E-25	2.24E-24
Ethyl formate	3727.2	1.77E-24	6.77E-24
Ethyl butanoate	3647.9	2.10E-24	7.42E-24
Hexanoic acid	3353.5	4.11E-24	1.35E-23
2-phenylethanol	3271.4	5.01E-24	1.46E-23
Ethyl acetate	3264.7	5.09E-24	1.46E-23
Amyl acetate	2648.1	2.71E-23	7.34E-23
Butyrolactone	2425.8	5.46E-23	1.40E-22
Acetic acid	2109.7	1.67E-22	4.04E-22
Heptan-2-ol	1849.6	4.77E-22	1.10E-21
Ethanol	1725.9	8.29E-22	1.82E-21
Pentan-2-one	1548.8	1.97E-21	4.11E-21
Butanol	1479.2	2.84E-21	5.68E-21
Acetoin	1301	7.92E-21	1.52E-20

Nonan-2-ol	1106.9	2.87E-20	5.29E-20
4-Heptanone	1100.6	3.01E-20	5.32E-20
Hexyl butanoate	1074.8	3.64E-20	6.19E-20
Propanol	842.4	2.54E-19	4.17E-19
Heptanol	783.53	4.52E-19	7.17E-19
Decanoic acid	603.69	3.61E-18	5.53E-18
Nonanal	501.51	1.58E-17	2.34E-17
Heptan-2-one	393.4	1.09E-16	1.56E-16
Dodecanal	379.61	1.44E-16	2.01E-16
Hexan-2-one	279.63	1.63E-15	2.20E-15
Butyl butanoate	252.4	3.66E-15	4.81E-15
Nonan-2-one	162.92	1.16E-13	1.48E-13
Hexanal	156.12	1.62E-13	2.01E-13
Undecan-2-one	122.14	1.11E-12	1.34E-12
Decalactone	111.65	2.23E-12	2.63E-12
Pinene	83.049	2.22E-11	2.55E-11
Decanal	73.825	5.49E-11	6.16E-11
Octanoic acid	56.567	4.21E-10	4.61E-10
2-Methyl propanoic acid	24.913	1.82E-07	1.95E-07
Benzaldehyde	22.109	4.25E-07	4.44E-07
3-Methylbutanol	3.6508	0.015146	0.015483

Table 3-5 Extraction of different volatile compounds by HS-SPME in 30 days ripening of Camembert cheese

3.4.1.3 Carboxylic acids

Carboxylic acids may originate from three main biochemical pathways during cheese maturation: lipolysis, proteolysis and fermentation of lactose (Curioni and Bosset, 2002). Lipolytic activity enzymes (esterases, lipases) may cause the release of linear-chain acids (butanoic, pentanoic, hexanoic, heptanoic, octanoic, decanoic and dodecanoic acids), while proteolytic enzymes are responsible for the formation of branched-chain acids (2-methylpropanoic and 3-methylbutanoic acids) by amino acids deamination, such as valine and leucine (Curioni and Bosset, 2002; Delgado et al., 2010). In this case, since the Camembert cheese is made from pasteurised milk, lipolysis could not originate from the milk through the action of indigenous lipases, but this is the responsibility of starter cultures. Finally, lactose fermentation with the growth of lactic acid and propionic bacteria could have given rise to acetic and propionic acids as changes in these acids have been previously associated (Ziino et al., 2005).

Most acids detected in curd came from proteolytic activity, while no acids had their origin in lipolytic activity or microbial degradation. However, the amount of acids derived from lipolysis tended to increase during ripening. During the first 10 days of maturation, carboxylic acids from amino acids increased and then slightly decreased until day 30. During ripening, acids with lipolytic origin increased significantly, so at day 30; they were the most abundant compounds, followed by acids derived from amino acids at the end of ripening.

The high amount of acids derived from amino acids may be associated with this cheese's high proteolysis during ripening (Delgado et al., 2010). Carboxylic acids were one of the most abundant volatile compounds isolated (17% of the total compounds) in the headspace of Camembert cheese on all days of ripening. They are also precursors of other compounds and not only aroma compounds by themselves such as methyl ketones, alcohols, lactones, aldehydes and esters (Collins et al., 2003a). Short and medium-chain carboxylic acids are considered important contributors to the flavour profile of a wide variety of cheeses due to their low aroma thresholds (Delgado et al., 2010; Kraggerud et al., 2008; Moio and Addeo, 1998; Pinho et al., 2003; Tavaria et al., 2004). At the beginning of maturation, the most abundant acids were acetic, propanoic, and 2-methylpropanoic acids. However, at day 1, high amounts of acetic acid and 2-methylpropanoic acid were isolated. Acetic acid content significantly increased after 5 days of ripening and it was one of the major compounds isolated in the headspace of Camembert cheese (See Figure 3.12). The content of acetic acid, butyric acid, 3-

methylbutanoic acid, octanoic acid, decanoic acid and hexanoic acid all significantly ($P < 0.05$) increased during the ripening. Decanoic acid was constant the first days of ripening and then tended to increase after 20 days of ripening. However, the content of 2-methylpropanoic and propanoic acid tended to decrease at the first days of ripening and then was constant during the ripening (See Figure 3.11). These changes are probably due to their transformation into other compounds, such as esters.

Acetic acid is the main free alkanoic acid in Camembert, produced by the action of lactic acid bacteria from different reactions (Delgado et al., 2010). Urbach (1993) and Dimos et al. (1996) found a steady increase in acetic acid levels throughout the entire maturation period. Another major acid detected 3-methylbutanoic acid, originated in the amino acid leucine breakdown (Kuzdzal-Savoie, 1980) and provided a rancid, cheese and sweaty odour (Yvon and Rijnen, 2001b). This compound may also be derived from aldehyde 3-methylbutanal oxidation. Propanoic acid formed at high concentrations originates in the lactate metabolism by *Propionibacterium* sp (Steffen et al., 1999) whereas 2-methylpropanoic acid is produced by the the amino acid valine metabolism (Molimard and Spinnler, 1996; Urbach, 1997a). Both propanoic as well as 2-methylpropanoic acids contribute to cheese aroma with sour odour notes (Avsar et al., 2004).

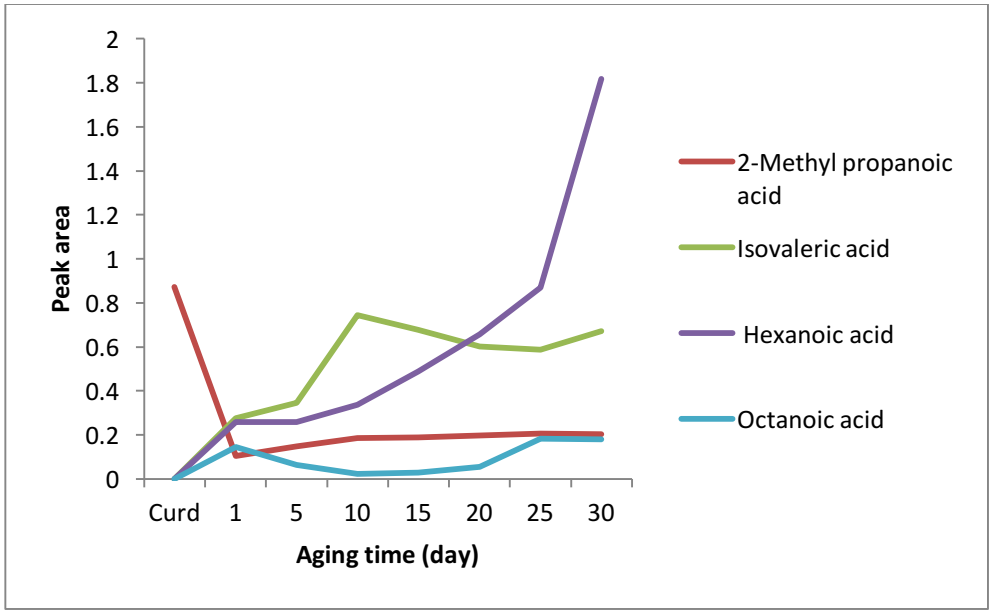


Figure 3-11 Changes of the main carboxylic acids isolated in the Camembert cheese during ripening in this study

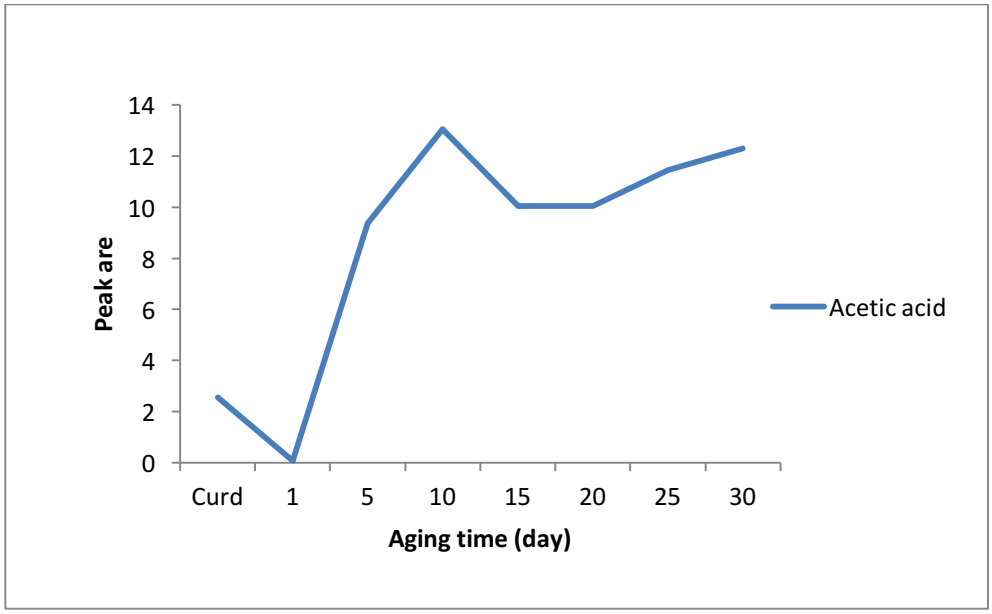


Figure 3-12 Changes of the Acetic acid isolated in the Camembert cheese during ripening

Flavour notes of some volatile fatty acids encountered in Camembert cheese at different time of ripening are shown in Table 3.6.

Acids	**Flavour note** (Lee et al., 2003)	**Aging time (day)**
Acetic acid	Vinegar, Pungent	Curd, Cam1, 5, 10, 15, 20, 25, 30
Propionic acid	Vinegar, Pungent	Curd, Cam1, 5, 10, 15, 20, 25, 30
2-Methyl propanoic acid	Sweet, applelike, rancid, butter	Curd, Cam1, 5, 10, 15, 20, 25, 30
Butyric acid	Rancid, cheesy	Cam 20, 25, 30
3-methyl butanoic acid	Rotten fruit, mild, fruity, sweet	Cam 1, 10, 20, 25, 30
Hexanoic acid	Pungent, blue cheese	Cam1, 5, 10, 15, 20, 25, 30
Octanoic acid	Wax, soap, goat, musty, rancid, fruity	Cam1, 5, 10, 15, 20, 25, 30
Decanoic acid	Rancid	Cam 5, 20, 25, 30

Table 3-6 Flavour notes of some volatile fatty acids found in Camembert cheese

3.4.1.3 Ketones

Ketones were the other abundant constituents found in the headspace with 21% of the total compounds isolated during ripening of Camembert cheese. They have typical odours and low perception thresholds. Pentan-2-one, nonan-2-one, heptan-2-one and acetoin were the most abundant ketones detected during 30 days ripening of Camembert cheese (See Figure 3.13). Pentan-2-one and hexan-2-one were not detected at early stage of ripening, but they greatly increased after 5 to 10 days of ripening, so they could play an important role in the final aroma of the Camembert cheese. Similar results have been found in other studies such as Adda et al. (1978) and Molimard and Spinnler (1996). Hexan-2-one was constant during ripening but slightly increased after 25 days. The opposite trend was found for 3-hydroxy- 2-butan-one (acetoin) which increased at the beginning of ripening but then decreased after 15 days and then was constant to the end of ripening.

Acetone is the major volatile compound in the curd's headspace, which was observed to decline with maturation due to its reduction to propan-2-ol, probably due to adventitious bacteria (Lecanu et al., 2002). Keen et al. (1974) showed that starter culture bacteria produce diacetyl, which is reduced to acetoin, and some starters can also reduce acetoin to 2,3-butanediol, while butanone and butan-2-ol production from 2,3-butanediol is caused by adventitious bacteria. While Acetophenone and Acetone showed close association to the curd, Undecan-2-one, Octan-2-one and 4-Heptanone had a positive correlation with the more matured Camembert.

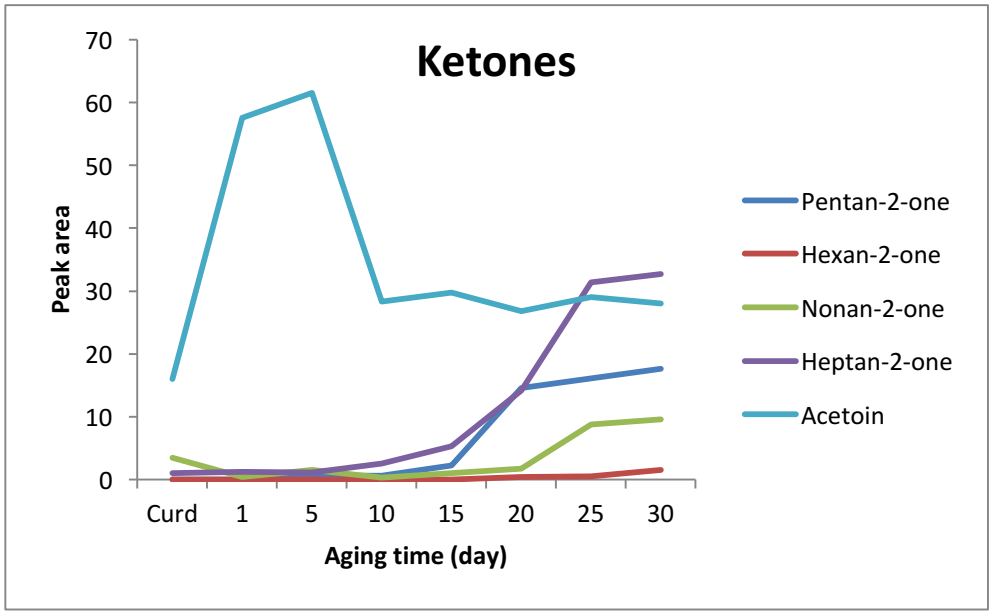


Figure 3-13 Changes of the main ketones isolated in the Camembert cheese during ripening

Flavour notes of some ketones encountered in Camembert cheese at different time of ripening are shown in Table 3.7.

Ketones	**Flavour note** (Lee et al., 2003)	**Aging time (day)**
Pentan-2-one	Fruity, acetone	Cam 5, 10, 15, 20, 25, 30
Hexan-2-one	Floral, fruity	Cam 15, 20, 25, 30
Undecan-2-one	Floral, herbaceous	Cam 20, 25, 30
Nonan-2-one	Fruity, musty	Curd, Cam 1, 5, 10, 15, 20, 25, 30
Octan-2-one	Fruity, musty	Cam 25, 30
Heptan-2-one	Blue cheese, spicy, Roquefort cheese	Curd, Cam1, 5, 10, 15, 20, 25, 30
4-Heptanone	Fruity	Cam20, 25, 30
Acetoin	Buttery	Curd, Cam1, 5, 10, 15, 20, 25, 30
Acetophenone	Orange blossom, floral, sweet	Curd
Acetone	Ethereal, powerful, fruity	Curd

Table 3-7 Flavour notes of some volatile ketones encountered in Camembert cheese

3.4.1.4 Alcohols

Primary and secondary alcohols, along with ketones, are considered to be the most important compounds in the volatilome of Camembert cheese (21% of total volatile compounds). The volatilome may be defined as all of the volatile metabolites that originate from an organism. Some definitions also include other volatile (organic and inorganic) compounds from the sample but in this thesis I will define it to refer to biological metabolites only. 3-methylbutanol, one of the primary alcohols was present in relatively large quantities in Camembert cheese samples tested and gives an alcoholic, floral note (Molimard and Spinnler, 1996). The principal secondary alcohol encountered was 2-heptanol, which presented similarly to the methyl ketones from which it was derived and made up 10 to 20% of all the volatile compounds in

Camembert cheese. Levels of 2-heptanol increased from the first day of ripening and it reached its maximum level at 15 days of maturation. It then decreased in concentration until the end of the ripening process. A similar trend was seen for appeared for 2-ethylhexanol, a compound known for its slightly sweet floral odour. This compound was also highest in the 15 day old sample, but was present in lower amounts compare to 2-heptanol. A related compound, 3-methylbutanol, which has with fruity and alcohol flavour notes also increased in concentration after the first week of ripening and reached its maximum level after 25 days (See Figure 3.14).

Octan-3-ol is well known for its raw mushroom odour. This gives a characteristic smell and taste to Camembert. Indeed, this compound is without doubt one of the key compounds in the global aromatic flavour of this cheese. It presented in small amount in young Camembert, since it is produced by *P. Camemberti*, and only appeared in higher amounts later in the ripening process. It is likely thus compound is was the result of the secondary metabolism as postulated by Spinnler et al. (1992).

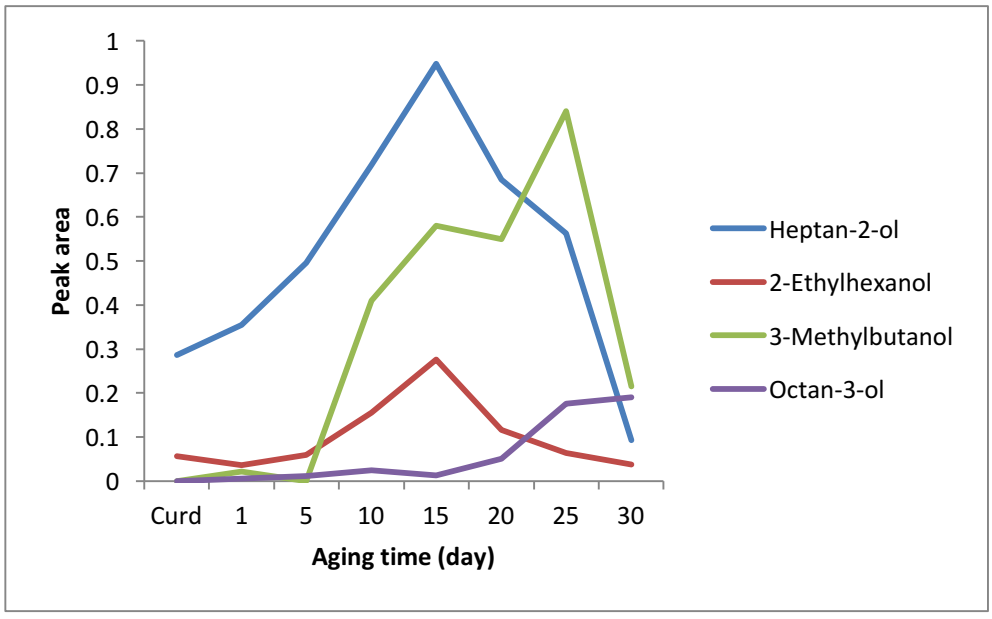


Figure 3-14 Changes of the main alcohols isolated in Camembert cheese during ripening

Flavour notes of some alcohols encountered in Camembert cheese at different time of ripening are shown in Table 3.8.

Alcohols	**Flavour note** (Lee et al., 2003)	**Sample**
Propanol	Mild, ether	Cam 30
Heptanol	Fragrant, oily, heavy, woody	Curd
Heptan-2-ol	Mild, ether	Cam 10,15, 20, 25, 30
2-Phenylethanol	Rose, floral	Cam15
2-Ethylhexanol	Sweet floral	Curd, Cam 15, 20, 25, 30
Nonan-2-ol	Green	Cam 30
3-Methylbutanol	Fruity, alcohol	Cam 10, 15, 25, 30
Ethanol	Alcohol, mild	Curd
Butanol	Sweet, fruity	Curd
Pentan-2-ol	Mild, ether	Cam1, 20, 30

Table 3-8 Flavour notes of some volatile Alcohols encountered in Camembert cheese

3.4.1.5 Lactones

Lactones are cyclic esters where acid and alcohol functional groups are present on the same molecule. In Camembert cheese, it has been previously suggested that triglycerides (normal constituents of milk fat) are subjected to hydroxy-fatty acid hydrolysis, followed by lactonisation (Spinnler et al., 1992). Additional mechanisms were also suggested by Adda et al. (1978) but none were ever proven.

Due to their fruity notes and their low perception thresholds, the presence of different γ and δ-lactones in Camembert may be important in the final aroma. δ-decalactone one of the most common and important lactones, also found in our case, at the end of maturation, in small quantities (See Figure 3.15). These results indicate that δ-decalactone associated with pronounced peach, apricot and coconut odour qualities, is a key compound that makes up the odour of mature Camembert cheese.

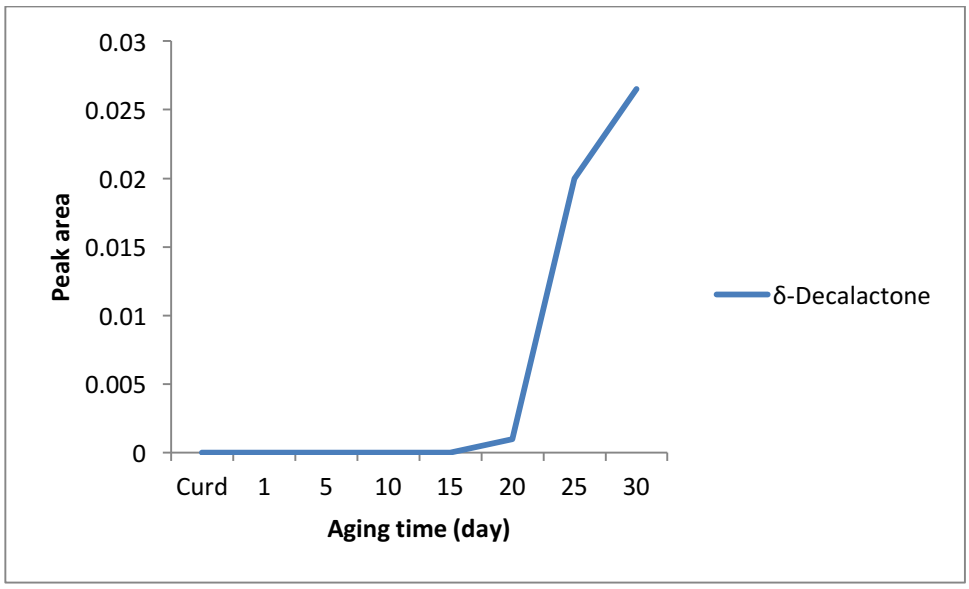


Figure 3-15 Changes of the δ-decalactone isolated in the Camembert cheese during ripening

3.4.1.6 Aldehydes

Aldehydes originate either through transamination from amino acids, leading to a decarboxylable intermediate imide, or through Strecker degradation (Keeney and Day, 1957). This reaction is simple and can even happen during maturation without enzyme catalysis (Keeney and Day, 1957).

Aldehydes were found to be transitory compounds in Camembert and other cheeses due to their rapid reduction to primary alcohols or even oxidisation to the corresponding acids. Branched-chain aldehydes are likely to originate from degradation of amino acids through enzymatic processes (Curioni and Bosset, 2002) and nonenzymatic reactions (Strecker degradation) (Keeney and Day, 1957).

The main aldehydes encountered in Camembert cheese are hexanal, benzaldehyde, octanal, nonanal, decanal and dodecanal. These compounds were presented in higher amounts in fresh curd but dropped to trace levels as soon as the first week of ripening (See Figure 3.16).

Hexanal gives the green note of ripe fruit, however, octanal, nonanal, decanal and dodecanal are described as having orange-like aromatic note. Benzaldehyde has been described as having a bitter almond aromatic note (Molimard and Spinnler, 1996). It seems likely that these compounds are not important to the final flavour of mature Camembert since they do not persist in the mature product. Higher than trace levels of these compounds in Camembert older than a week may indicate issues with the maturation process and would negatively impact flavour.

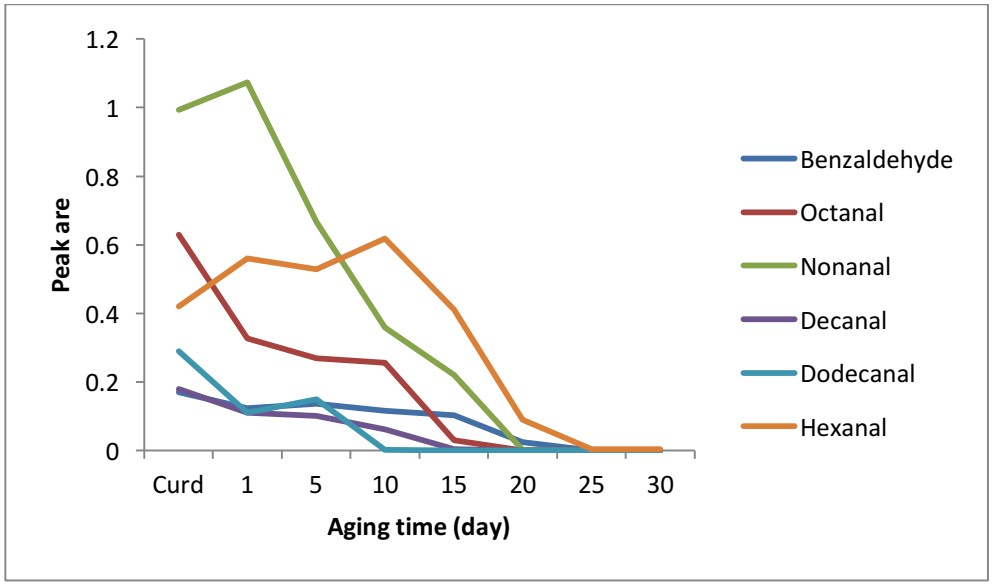


Figure 3-16 Changes of the main Aldehydes isolated in the Camembert cheese during ripening

3.4.1.7 Esters

Esters were found to be common members of the Camembert volatilome. Esterification reactions happen between fatty acids of the short- to medium- chain and primary and secondary alcohols from lactose fermentation or catabolism of amino acids (Kubícková and Grosch, 1998). The microorganisms involved in the formation of esters are primarily yeasts (Molimard and Spinnler, 1996); some lactic acid bacteria and even some minor chemical reactions may also be responsible (Delgado et al., 2010). The action of the last two seems to be less probable in the Camembert cheese given the large amount of biological activity in the system.

Most esters are described as having sweet, fruity and floral notes. Ethyl esters in cheese are known for their important role in creating a fruity character. Some esters, like hexyl butanoate and ethyl acetate have a very low perception threshold. These compounds may be contributing to the aroma of Camembert cheese by minimising the sharpness and bitterness, which are characteristic flavours of fatty acids and amines (Kubíčková and Grosch, 1997). Our results indicated that Ethyl butanoate, isoamyl formate and butyl butanoate are the most important aromatic esters in Camembert cheese, although butyl butanoate is present in quite low concentrations. Changes in ethyl butanoate, isoamyl formate and butyl butanoate levels during ripening of Camembert are shown in Figure 3.17.

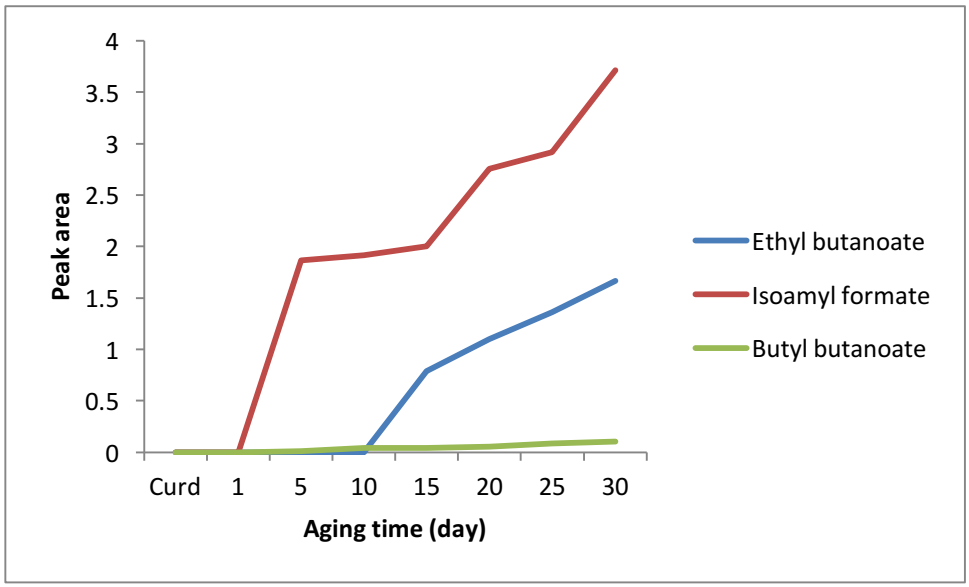


Figure 3-17 Changes of the main Esters isolated in the Camembert cheese during ripening

3.5 Conclusion

As the Camembert matured, a greater amount of aroma volatiles, especially acids 17% (such as acetic, 3-methyl Butanoic, hexanoic, 2-methyl propanoic and octanoic acids), ketones 21% (like heptan-2-one, nonan-2-one, pentan-2-one and acetoin), alcohols 21% (such as octan-3-ol, 3-methyl butanol and heptan-2-ol) and esters 15% (like Ethyl butanoate, isoamyl formate and butyl butanoate) were produced. In addition, the older Camembert cheese showed cheesier and fattier profiles with higher concentration of characteristic aromas. The overall effect of maturation resulted in a gradual increase in aroma of the cheese and it was possible to see what was causing this change.

The HS-SPME technique was shown to be useful for the successful monitoring of changes in the volatilome of Camembert composition. A number of flavour compounds from multiple checial classes were able to be identified and monitored for through the maturation process and without damaging the samples (since only headspace analysis was conducted). HS-SPME has therefore been shown to provide a satisfactory evaluation of changes of the cheese during maturations and also to provide a good fingerprint of the compounds which are responsible for consumer odour perception.

Chapter 4 NEAR INFRARED SPECTROSCOPY (NIR)

4.1 Introduction

The drive towards better quality control of raw materials and finished food products requires the use of precise, fast and efficient methods to analyse food components. The food industry now also needs to have tools available for real-time control of production lines to check whether in-process material meets the compositional or functional specifications necessary to achieve a predetermined quality standard in the final product during a given processing step (Blazquez et al., 2004). NIR spectroscopy is a widely analytical tool in food production in this context. This method provides quick and reliable quantitative determination of moisture, fat, protein, sugars and other organic components (Adamopoulos et al., 2001). NIR spectroscopy was used for the determination of moisture in corn kernels by Finney and Norris (1978). Determination of protein in wheat samples was another significant application of NIR (Osborne and Fearn, 1983). NIR as a technique has been used not only as a tool for quality control but also for on-line measurements in food industry over the past two decades (McClure, 1994). In the case of dairy products, NIR analysis may be used for to determine fat, moisture, lactose and protein in milk samples, but has not been reported in soft cheese before (Baer et al., 1983).

The NIR spectrum of milk was studied by Bengera and Norris (1968) to determine fat and moisture content. The authors applied multiple linear regressions (MLR) to the data, laying the foundation for the development of a correlation-based calibration equation. Since the Bengera and Norris study much work has been published to determine the chemical composition of milk, Robert et al. (1987), and milk powders Casado et al. (1978) and Frankhuizen and Van der Veen (1985). The latter was able to develop a classification system of milk powders based on heat treatment.

Downey et al. (1990) studied the analysis of cheese macro-components by NIR spectrophotometry. Since that time, research has been undertaken for developing NIR calibrations to predict cheese constituents like fat, protein and total solids (Cepeda, 1995) as well as to monitor production processes (Adamopoulos et al., 2001). In the context of increased production of cheese and processed cheese, NIR meets the dairy industry's needs for a rapid monitoring technique that can improve plant versatility and produce high quality products at

minimal cost (Blazquez et al., 2004). However, little work on the application of NIR to processed cheese analysis have been reported to date.

Overall, cheese accounts for around 30% of total dairy product sales with a sales growth of 9.8% between 2003 and 2007 so, it is important to quickly and cost-effectively determine cheese quality (Woodcock et al., 2008). Traditionally, different physico-chemical methods have used the chemical characteristics of cheeses to determine pH, fat content, nitrogen fractions, volatile fatty acids, organic acids, etc (Karoui et al., 2007). These methods can be labour-intensive and costly. In addition to the need for efficiency, there is an emerging need for food processing to be determined online and in real-time for all major compositional and quality parameters. To meet these needs, the potential of techniques such as infrared spectroscopy, ultrasound, and computer vision was examined. It is also necessary for food industries to show the authenticity of their products (Woodcock et al., 2008). Another important issue related to on-line NIR analysers is the ability to gain data in real time and allow for adjustment to the system to correct for them. This is in stark contrast to the GC-MS methods in the previous chapter for example, which require data to come back from the lab before anything can be changed in production, by which time it may well be too late.

This chapter reports a study examining the possibility of assessing, controlling and predicting the quality of Camembert during ripening with NIR spectroscopy. Determinations of moisture, fat and protein were selected as the NIR analyser's calibration set. The calibration data resulting from this work can be used for Camembert cheese production on-line quality control.

4.2 Overview of NIR Spectroscopy

NIR radiation is defined as the wavelength region between the visible light and the infrared light between 750 and 2,500 nm (Büning-Pfaue, 2003). NIR spectroscopy is a high-precision physical, non-destructive technology that requires minimal or no preparation of samples. It is also suitable for use online. Once calibrated, with minimal training, an NIR spectrometer can be operated. There are two dominant and wide peaks in a typical NIR food spectrum near 1,440 and 1,930 nm. These peaks are caused by water and are responsible for certain typical complications in later chemometric analysis. The reliability of NIR spectroscopic results is also affected by the effects of hydrogen bonding and sample temperature (Büning-Pfaue, 2003).

NIR spectroscopy's main disadvantage is its weak sensitivity to minor components such as salt and water-soluble nitrogen.

For most constituents, the sensitivity limit of NMR is about 0.1 percent (Iwamoto and Kawano, 1992). The development of food industry instruments, measuring techniques, and chemometrics has been widely reviewed so is not commented on in depth here (Bakker et al., 2012; Benson, 2003; González-Martín et al., 2011; Penner, 2017; Wang et al., 2017; Woodcock et al., 2008). Traditionally, NIR spectroscopy has been used to measure the compositional parameters of food products. However, it may also be used to determine complex quality properties such as texture and sensory attributes (Woodcock et al., 2008). Examples of matured Camembert cheese NIR spectra are shown in Figure 4.1.

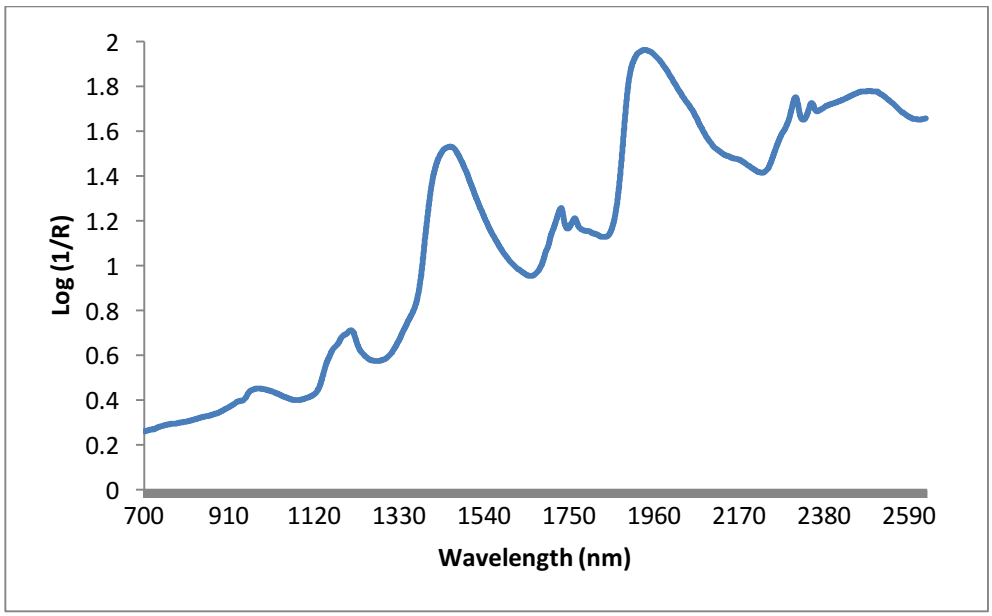


Figure 4-1 NIR spectra of matured Camembert cheese between 700 and 2600 nm

As discussed in chapter one, during the maturation and aging, cheese undergoes a complex series of chemical, bacterial, and enzymatic reactions that are responsible for breaking down the protein matrix and ultimately for developing the texture and sensory characteristics typical of mature cheese. Therefore, it is important to determine the degree of maturation or aging of cheese and is important, and as stated by Martin-del-Campo et al. (2007), having available information on the evolution of lipolysis, glycolysis, and proteolysis may help the cheese industry to better understand the biochemical kinetics of maturation in order to improve the aging process and possibly even speed it up (as was recently demonstrated for rum -

https://www.wired.com/2017/05/brian-davis-lost-spirits-distillery-aging-rum-fast/). There is considerable interest in developing instrumental techniques to allow for more objective, faster and less expensive evaluations in this area (Downey et al., 2005) but this work has not yet been undertaken to any great extent. This means that many methods for determining even comparatively simple things such as fat and moisture content require time consuming and expensive lab tests, which do not give instant feedback. The use of NIR might be able to provide the same data much faster and thus has high potential for impact in the industry if it could be shown to work reliably. This is the aim of this chapter.

It should be noted that the wavelength used to analyse cheese composition varies widely. Many authors use the full NIR spectra of 400–2,500 nm . (Blazquez et al., 2004; Cepeda, 1995; Pérez-Marín et al., 2001; Wittrup and Nørgaard, 1998). Some authors like as Adams et al. (1999) used a low range in the NIR spectrum, while others like Lee et al. (1997) used a higher range (1,900–2,320 nm). After a close study of the literature it was found that, while the results depended on a number of parameters and varied from study to study, the general trend in the published papers showed that the best results were found when the full NIR spectrum (400–2,500 nm) was used to analyze cheese composition and so this was what was done for this thesis.

4.3 Material and methods

4.3.1 Sampling of Camembert cheese

Spectroscopic data were collected in reflectance mode between 700 and 2800 nm using a PE spectrum 10 software (PE Dairy Guard), fitted with a PE Frontier NIR CaF_2 beam splitter. Because of the effect of temperature on the spectral response of high moisture samples (Wehling and Pierce, 1987), cheese samples were equilibrated to room temperature (20-24 °C) prior to NIR analysis. This was achieved by removing them from 4°C storage and leaving them on the laboratory bench for 2h.

Three cylinders (0.5 cm height and 3.8cm diameter) of cheese were removed from each cheese block with the aid of a knife. Each of these samples was scanned in duplicate with rotation of the sample cup through approximately 120° between successive scans. Subsequently, the average spectra of each cheese sample were used to develop the calibration models. The value

of the root mean square *(RMS)* between the replicate scans will be less than 3000 micro-absorbance units for any given sample. Data was recorded as log 1/R, where R is the energy of reflection.

4.3.2 Preliminary studies

In order to have some data to judge the NIR results against a range of parameters were first obtained from the cheese using standard measures.

4.3.2.1 Total fat

Soxlet extraction was undertaken to measure total fat content. First, 500ml round bottom Soxhlet extraction balloons were brought to constant weight by drying in the drying oven at 105 ℃ for 1 hour. Next 5 g of ground cheese were weighed out with an analytical balance. These samples were then placed in 500 mL Erlenmeyer flask, with pieces of pumice stone as boiling regulators and 100 mL of 4 mol/L hydrochloric acid solution. The mixture then underwent 1 hour of smooth boiling. With the help of a cotton pad soaked in 250 mL n- hexane (pesticide grade), the mixture was then transferred in quantity on filter paper. The cotton was placed on the thimble of cellulose (22 × 88 mm) and introduced in the main chamber of the Soxhlet extractor. Then 250 ml n- hexane (pesticide grade) was added to the balloon and the extraction was performed under reflux for 6 hours. The solvent was released in a rotary evaporator after extraction and the last traces were removed by placing the flask with the extract in an oven at 100 °C for 1 hour, followed by cooling in a desiccator and weighing. This last step was repeated until the weight difference between repeated measurements was <10 mg (Chis and Purcarea, 2009).

Equation 1 was been used for the calculation of the mass fraction of fat of the sample:

$$Fat\% = (m_2 - m_1)/m_0 \times 100 \qquad \text{Equation 1}$$

m_0= the mass of the test portion, g

m_1= the mass of the empty flask, g

m_2= the mass of the fat-extracted flask, g

4.3.2.2 Moisture content

Moisture was determined using the standard AOAC 926.08 (1990) method but replacing the vacuum oven with an air oven. Moisture content was calculated as the weight loss, expressed as a percentage, when cheese samples were dried at 100°C in oven overnight (about 16 hours). It can be seen that even this comparatively simple test took a long period of time to provide data.

Fine-cut and mixed samples of cheese (5 g) were precisely weighed and dispersed in an aluminium dish in a uniform layer. Dried samples were removed from the oven, cooled and weighed in a desiccator at room temperature (about 1 hour). During the cooling phase, a lid was placed on top of the moisture plates. Analysis was conducted in duplicate with the mean being taken as the final outcome. The total moisture content was determined for each sample using **equation 2**.

$$\text{Total moisture (\%)} = \frac{(C-D)}{C} \times 100 \qquad \text{Equation 2}$$

C= Initial weight of the grated cheese sample drying (g)

D= Final weight of the grated cheese sample following drying (g)

4.3.2.3 Total and soluble nitrogen

Total nitrogen was measured using Kjeldahl determination as outlined previously (Kosikowski and Mistry, 1982). One half gram of well blended sample was placed into a Kjeldahl digestion tube with 0.19 grams $CuSO_4$, 0.11 grams pumice and 20 ml of concentrated H_2SO_4. Samples were then digested for 3 hours at a temperature of 400° C and then cooled.

The digested samples were then neutralized with 150 ml of a 30% NaOH solution. The ammonia was distilled into flasks containing 50 ml of a 4% boric acid solution, methylene blue and methylene red indicators. The ammonia in the boric acid solution was then back titrated with a 0.1 N H_2SO_4 standardized solution. The nitrogen content was then calculated from the volume of 0.1 N H_2SO_4 needed to turn the solution from a green to a purple endpoint (Williams, 1984). The total nitrogen (TN) is defined as the amount of nitrogen and is measured by the Kjeldahl determination.

$$\%\text{Protein} = F \times \%N \qquad \text{Equation 3}$$

F=6.38

4.3.2.4 pH

The pH of the blended cheese sample was measured with a Beckman Zeromatic Model pH meter (Beckman Instruments, Inc., Fullerton CA) by immersing the electrode of pH meter into a blend of grated cheese (10 g) with 50 ml of distilled water (Kosikowski and Mistry, 1982).

4.3.2.5 Total solids

The total solids content was determined by drying duplicate 2 g of Camembert cheese samples in a vacuum oven at 50'C for 24 h under pressure (Schlesser et al., 1992).

4.3.2.6 Water activity (a_w)

Water activity (a_w) for the Camembert cheese samples was determined according to the method of Landrock and Proctor (1951) with saturated salt solutions of known a_w.

4.4 Results and discussion

4.4.1 Preliminary studies

Because of surface evaporation, total solids increased during ripening. Fat and protein calculated on a wet basis, followed the same trends as the total solids. The pH of the camembert cheese decreased from curd to day 1, and then increased from 3.8 to 5.5 by day 30. This increase may be explained by the assimilation of the lactic acid and the deamination of the amino acids by the mould. Thus, as the mould neutralized the acidity of the cheese, the pH increased (Schlesser et al., 1992).

Moisture content decreased during ripening, the initial lowering in moisture during ripening was due to surface evaporation of the water. After 10 days of ripening this surface evaporation was decreased. Further decrease in moisture content during this time may be attributed to

hydrolysis of the casein. The casein was converted to peptone, peptides, free amino acids, and even ammonia

Water activity decreased during 30 days of Camembert ripening. Schlesser et al. (1992) found that, as proteolysis increased, water sorption increased, the end of ripening occurred when the maximum amount of water was held or stabilized by the hydrophilic groups resulting from proteolysis.

Sample	Moisture	Fat	pH	Total solid	Protein	Water activity
Curd	48.71	13.91	6.7	36.2	16	0.98
Cam 1	38.28	20.05	3.8	40.83	15.5	0.94
Cam 5	37.02	21.02	4.1	41.6	17.4	0.91
Cam 10	36	21.07	4.2	45.55	19.8	0.89
Cam 15	33.74	24.07	4.6	51.34	20	0.86
Cam 20	32.92	27.44	4.9	48.7	18.7	0.81
Cam 25	30.87	26.43	5.2	50	21.1	0.76
Cam 30	29.91	26.38	5.5	52.3	19.2	0.59

Table 4-1 Mean analytical values (%w/w) for moisture, fat, pH, total solid, protein and water activity of Camembert cheese during ripening

4.4.2 Developing calibration model to predict moisture, fat, total solid and protein in camembert cheese using near infrared spectroscopy during ripening

Cheeses are produced with a variety of texture and parameters of composition. While the variety of cheese type is desirable, its texture, which in turn is influenced by moisture and other compositional components and processing conditions, greatly determines the quality of any, given type of cheese. Manufacturing have traditionally relied on a wide range of techniques for chemical analysis to quantify major food components such as moisture, protein, and fat (Woodcock et al., 2008).

NIR reflectance spectra of Camembert cheese at different times of ripening are shown in Figure 4.2. A number of different regions can be observed in the spectra. Three of these describe water region: 970 nm and 1450 nm (second and first overtone of the O–H stretch vibration), 1940 nm (a combination of O–H stretch and deformation). Bands at 1208 nm (a –CH stretch second overtone), 1728 and 1765 nm (first overtone of the –CH stretch) and 2310 plus 2345 nm (combined –CH stretch and deformation band) belong to fat components.

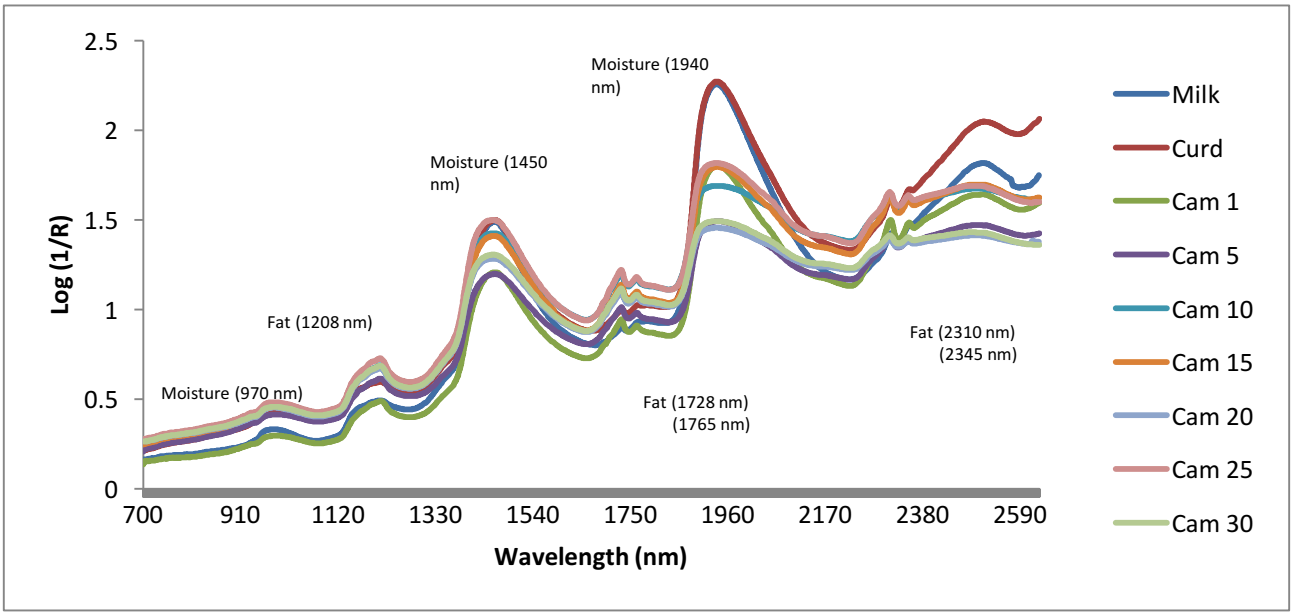


Figure 4-2 NIR reflectance spectra of Milk and Camembert cheese at different time of ripening

In total, 6 training set were developed for each of the four constituents (moisture, fat, total solid and protein). The correlation coefficient (*R*) and root mean square error (*RMS Err*) of PLS loadings for each of the training developed are shown in Table 4.2.

Correlation coefficients (*R*) obtained for moisture content were lower than those obtained for protein, total solid and fat, and ranged from as low as 0.29 to a maximum value of 0.99. Minimum and maximum correlation coefficients (*R*) obtained were 0.4 and 0.99 for fat, 0.7 and 0.99 for protein, and 0.45 and 0.99 for total solid, respectively. The number of PLS loadings for moisture, fat, total solid and protein developed with six loadings. The preferred moisture model produced with a root mean square error (*RMS Err*) equal to 0.56, used six PLS loadings and had a correlation coefficient (*R*) of 0.99. Data produced by this model are shown graphically in Figure 4.3.

Figure 4.4 shows the plot of six PLS loadings used for fat prediction in Camembert cheese. The preferred model for fat had a root mean square error (*RMS Err*) equal to 0.11, used six PLS loadings and had a correlation coefficient (*R*) of 0.99. The same trend was seen for protein (See Figure 4.5) and total solids (See Figure 4.6), obtained with a root mean square error (*RMS Err*) equal to 0.14 and 0.75 and correlation coefficient (*R*) of 0.99 for both of them. In the spectra there are six regions where the difference between the components can be seen. The main moisture regions can be observed at about 1000, 1450 and 1900 nm. The fat regions can be seen in the 1200, 1700 and 2300 nm.

Results were compared to those obtained by other published works for hard cheeses (as opposed to the soft cheese used here). Lee et al. (1997) reported, PLS regression models for fat and total solids prediction in processed cheese were obtained using reflectance spectra (1100–2200 nm) collected with a fibre optic probe on a NIR spectrometer. Their models yielded a standard error of prediction (*SEP*) of 0.997 and 0.429% w/w for fat and total solids, respectively, and correlation coefficients (*R*) of 0.995 and 0.998. In another study, Blazquez et al. (2004) investigated the application of several multivariate calibration methods including ordinary least squares (OLS), multiple linear regression (MLR), principal component regression (PCR) and partial least squares (PLS) regression for prediction of processed cheese composition. In this case, the best models to predict moisture and fat were obtained using principal component regression with *RMS Err* values of 1.25% w/w and 1.24% w/w for moisture and fat, respectively. These results were obtained using spectral data from a 12-filter spectrometer. Therefore, it's apparent that the results in the present study are significantly more accurate than those in previous reports.

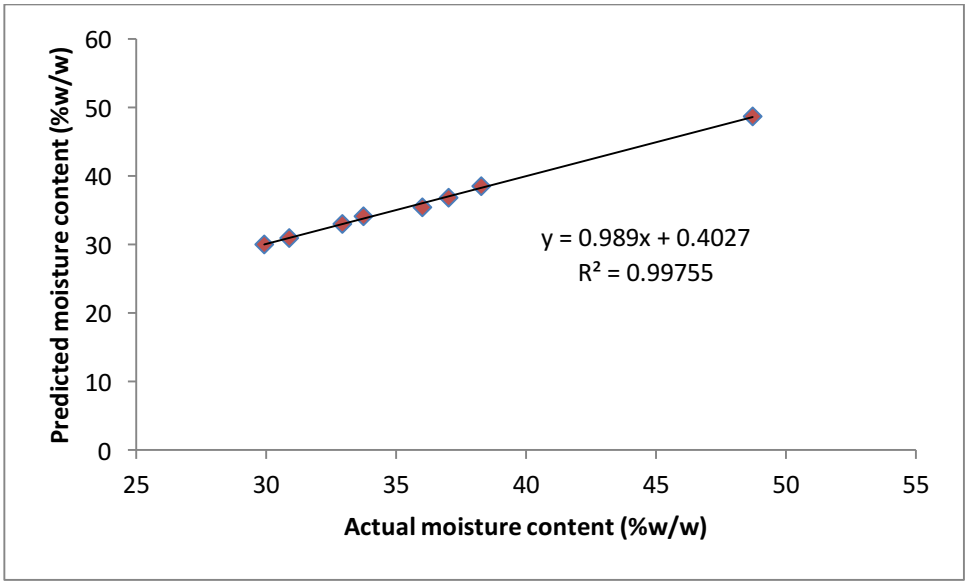


Figure 4-3 PLS regression of actual versus predicted moisture values (700-2600 nm)

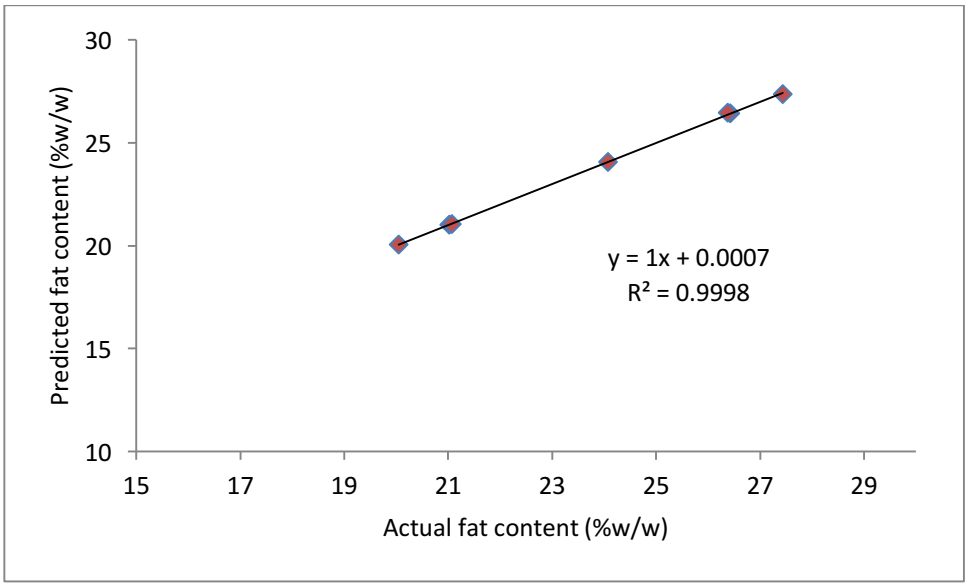


Figure 4-4 PLS regression of actual versus predicted fat values (700-2600 nm)

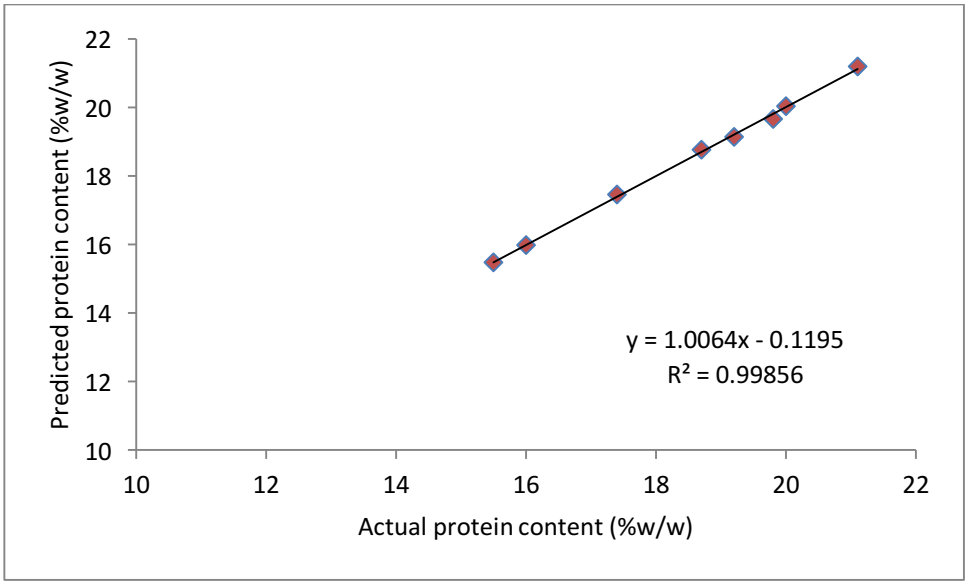


Figure 4-5 PLS regression of actual versus predicted protein values (700-2600 nm)

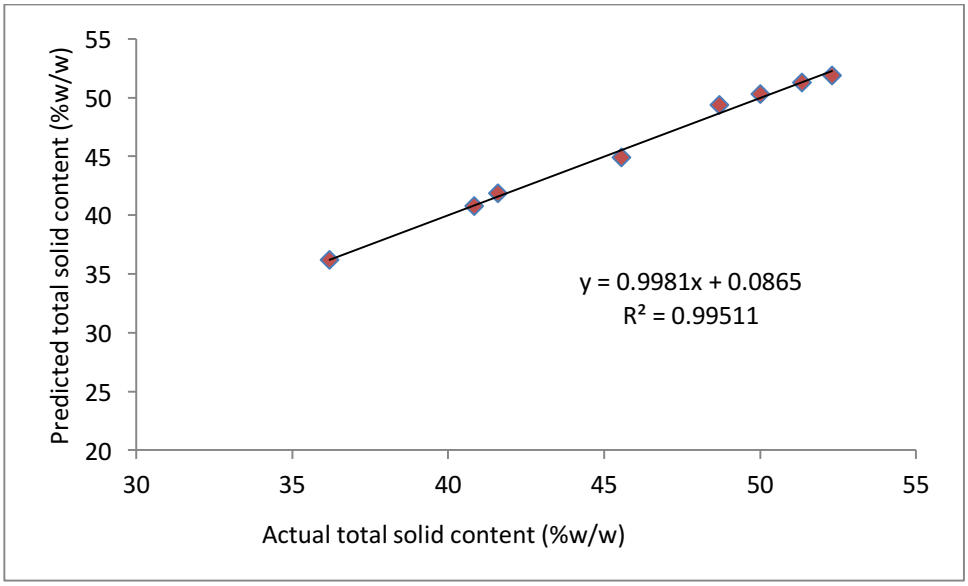


Figure 4-6 PLS regression of actual versus predicted total solids values (700-2600 nm)

	Training set 1		Training set 2		Training set 3		Training set 4		Training set 5		Training set 6	
	R^2	RMS Err	R^2	RMS Err	R^2	RMS Err	R^2	RMS Err	R^2	RMS Err	R^2	RMS Err
Moisture	0.29	10.48	0.29	6.12	0.43	6.26	0.86	3	0.97	1.5	**0.99**	**0.56**
Fat	0.51	2.97	0.40	3	0.65	2.25	0.96	0.94	0.99	0.31	**0.99**	**0.11**

Protein	0.77	1.67	0.70	1.35	0.83	1.09	0.95	0.74	0.99	0.31	**0.99**	**0.14**
Total Solid	0.48	5.59	0.45	4.48	0.65	4.40	0.97	1.15	0.98	0.96	**0.99**	**0.75**

Table 4-2 Summary results of moisture, fat, protein and total solid predictions in Camembert cheese during ripening

4.5 Conclusion

The NIR models developed were applicable for rapid analysis of Camembert cheese at various stages of ripening. NIR was shown to provide a routine, fast, and efficient method for simultaneously analysing moisture, protein, fat, and total solid in Camembert cheese that is faster than current methods. With rapid developments in NIR measurement, it becomes more feasible for continuously monitoring compositional changes during cheese making. Such measurements could provide an effective means for control of cheese quality during manufacturing. The results presented here show that the application of NIR spectroscopy could potentially improve the dairy production economy through the optimised laboratory efficiency, the increased product quality and the tighter production control.

Chapter 5 HIGH RESOLUTION NUCLEAR MAGNETIC RESONANCE (^{1}H-NMR) TO EVALUATE PROTEOLYSIS OF AMINO ACIDS IN CHEESE

5.1 Applicability to metabolomics

High resolution ^{1}H NMR spectroscopy is a relatively fast analytical method and is extremely robust in terms of results reproducibility. The ubiquity of protons in cell metabolites and the fact that NMR can observe other nuclei (e.g. ^{31}P and ^{13}C) means that a relatively large number of different metabolites can be detected. The technique also requires minimal sample preparation and the non-destructive nature of the technique allows further analysis to be carried out. ^{1}H NMR is, however, an inherently insensitive analytical tool that only measures metabolites of high concentration. The suitability and reliability of Nuclear Magnetic Resonance spectroscopy for contributing to metabolomic studies of food products has been identified repeatedly (Brescia et al., 2005; Gianferri et al., 2007; Kuo et al., 2003; Luykx and Van Ruth, 2008). However, prior to liquid-state NMR spectroscopy, preliminary and specific chemical extractions are commonly required, resulting in potentially time-consuming and expensive analyses.

5.2 Principles of NMR

The principles of NMR are discussed in section 2.8.1 so are not repeated here.

5.2 Material and methods

5.2.1 Sampling of camembert cheese

Cheese samples were sourced from LD&D Foods Pty Ltd. These cheeses were ripened at the fridge (2-4℃) for 30 days. Samples were again taken at deferent stages of ripening (curd, Cam day 1, Cam day 5, Cam day 10, Cam day 15, Cam day 20, Cam day 25 and Cam day 30). The samples were placed in special containers and kept in liquid nitrogen until used in order to maintain their characteristics and avoid degradation.

5.2.2 Extraction technique

A methanol-chloroform-water extraction of cheese samples was performed using the method previously described by Bligh and Dyer and subsequently modified by Miccheli et al. (1988).

A mixture of cold chloroform:methanol (1:2) was added in a ceramic mortar containing 5 g of frozen cheese samples in a liquid nitrogen bath. The samples were vortexed with 1 ml chloroform and 1 ml water for each gram of cheese sample. The phases were separated by centrifuging at 10,000 ×g for 20 min at 4°C and dried under a nitrogen flow. The extracts were dissolved in 0.4 ml deuterium oxide (D_2O-99.996%, Sigma Chemical Company) for ^1H NMR spectroscopy.

5.2.3 ^1H NMR Procedure

High-resolution experiments were performed using a Bruker AVANCE III-300 NMR spectrometer with an operating at a proton frequency of 300.1400 MHz.

All ^1H-NMR spectra were recorded at the usual working temperature of the instrument, i.e. 25 °C. Standard one-dimensional (1D) ^1H spectra were acquired using a 1D NOESY pulse sequence with 512 scans and acquisition time of 2.59 s. 16384 points were counted with spectral widths of 6313.13 Hz. Water suppression was achieved by irradiation of the water resonance with a weak field of radio frequency.

5.3 Data analysis

Data analysis performed with the Metaboanalyst software. The log transformation (generalized logarithm transformation or glog) and auto scaling (mean-cantered and divided by the standard deviation of each variable) were used as scaling methods to make individual features more comparable. Principal Component Analysis (PCA) was performed on all Camembert cheese samples used for NMR determination. The useful parameters achieved from the models were the variable influence on projection (*VIP*) scores and coefficients indicating the effect of metabolites on all validated components contributing to the separation of sample groups, and the comparative metabolite levels in each group (Eriksson et al., 2013). PCA data matrices were built from 10 to 0 ppm but excluding water. Note that the large amount of lactic acid parts of the spectra which are the result of the variation in pH that happens during maturation due to the LAB in the system.

The first three principal components were calculated for each model. A supervised partial least squares-discriminant analysis (PLS-DA) analysis was subsequently also performed on the data

for comparison with the PCA. The PLS-DA analysis provided three predictive components using no class information.

5.4 Results and discussion

High resolution NMR experiments allowed all the amino acids present in the Camembert cheese to be quantitatively and qualitatively evaluated as set out below

5.5 Evaluate the proteolysis (detect amino acids by ^{1}H NMR)

The complete ^{1}H spectrum of Camembert cheese during 30 days of ripening is shown in Figure 5.2. By comparison of the eight spectra, it can be seen that significant differences in the levels of amino acids occur during ripening, resulting in markedly different metabolic profiles. To make this data easier to see, Table 5.1 shows, the free amino acid contents as a percentage of total amino acids in Camembert cheese samples at deferent ripening stages curd, day 1, day 5, day 10, day 15, day 20, day 25 and day 30, respectively.

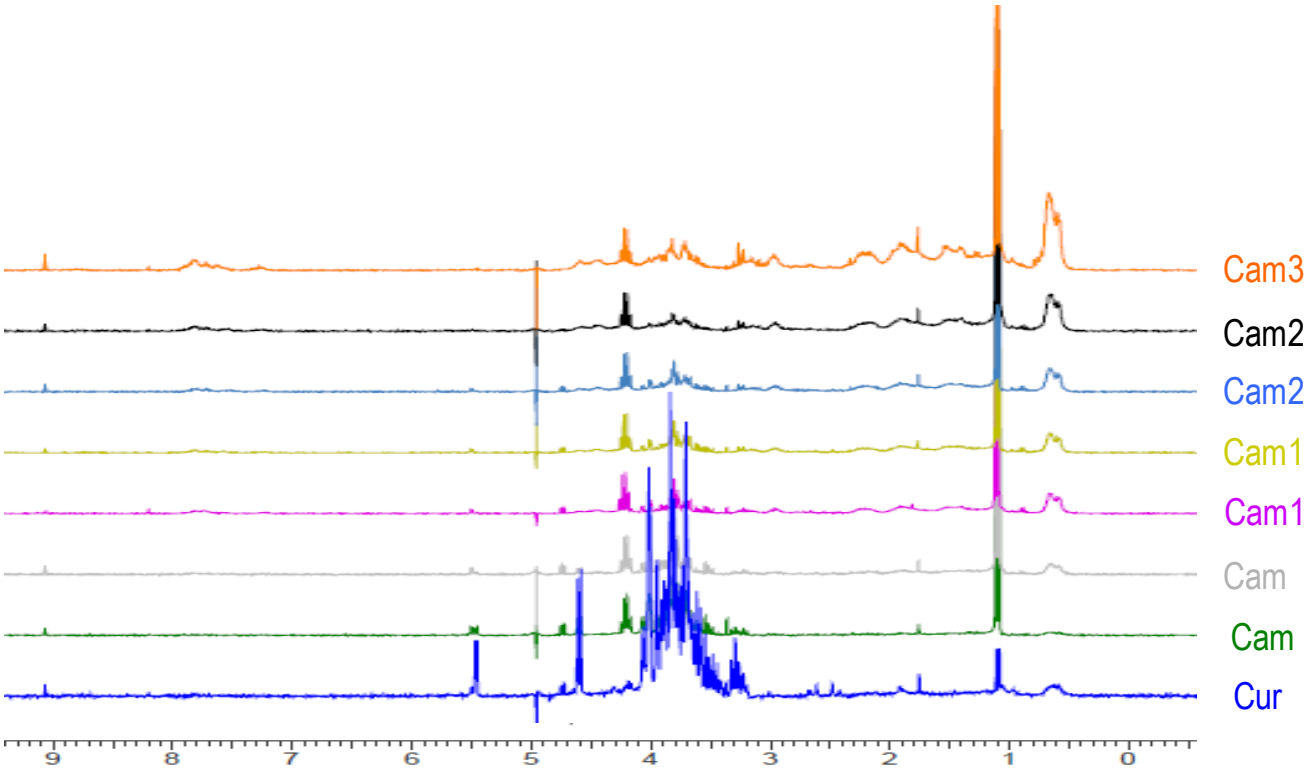


Figure 5-1 ^{1}H NMR spectrum of Camembert cheese during ripening

Figure 5.3 shows the ^{1}H NMR spectrum of Camembert cheese in the curd stage. Changes in abundances of 15 representative amino acids and also lactate levels over the ripening time are shown in Figure 5.4.

From the data it can be seen that, as a function of the ripening time, in particular, leucine (resonances at about 3.72-3.75 ppm), valine (resonances at about 3.6 ppm), phenylalanine (resonances at about 3.12 ppm), methionine (resonances at about 3.88 ppm), aspargine (resonances at about 2.93 ppm), and glutamic acid (resonances at about 3.76 ppm) increase, while threonine (resonances at about 3.57 ppm), proline (resonances at about 4.10-4.14 ppm), serine (broad resonances at 3.82-3.99 ppm), aspartic acid (resonances at about 4.7 ppm), and histidine (resonances at about 3.96 ppm) decrease. Such a variation is related to proteolysis and to other metabolic processes during the storage period and indicates the role played by the enzyme system present in the samples (Angelis et al., 2000).

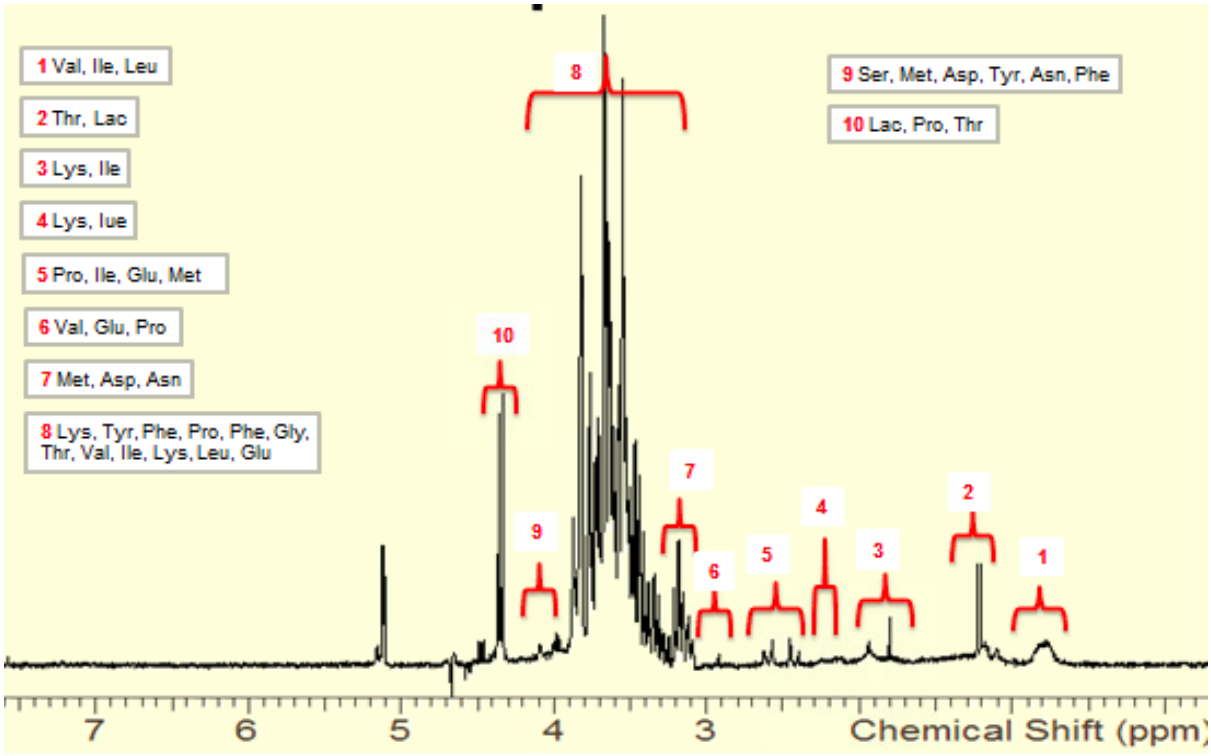
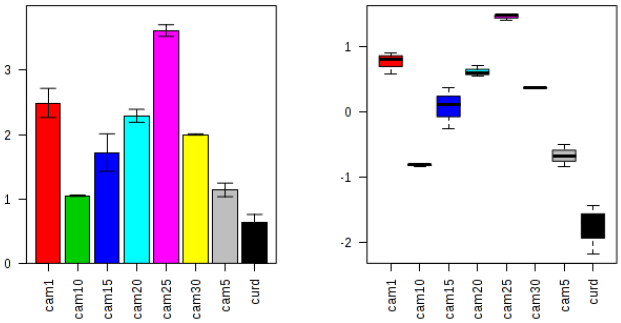


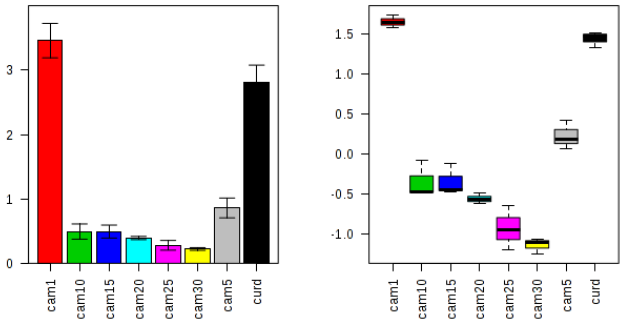
Figure 5-2 ^{1}H NMR spectrum of Camembert cheese at curd stage with complete spectrum and expected regions

The level of free amino acids in the curd sample was higher than in other samples. This confirms the amino acid conversion to aroma compounds by peptidases and proteinases activities in the cheese matrix during ripening. Several factors are likely to be responsible for the degradation of amino acids intensity; first is the number of aminotransferases active on each amino acid, the second is the affinity of aminotransferases for the different amino acids, the third is the respective intracellular concentration of each amino acid and the last is the further degradation of the transamination products like a-ketoacids (Yvon et al., 1998).

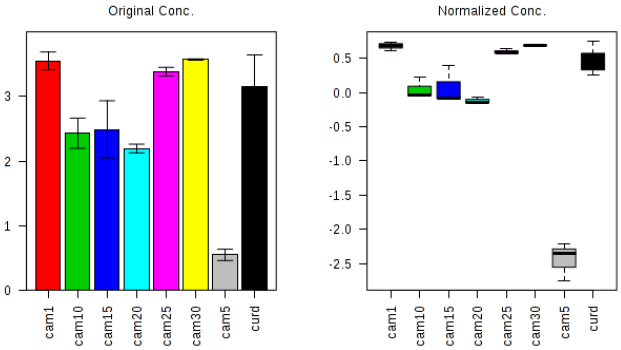
Aspargine



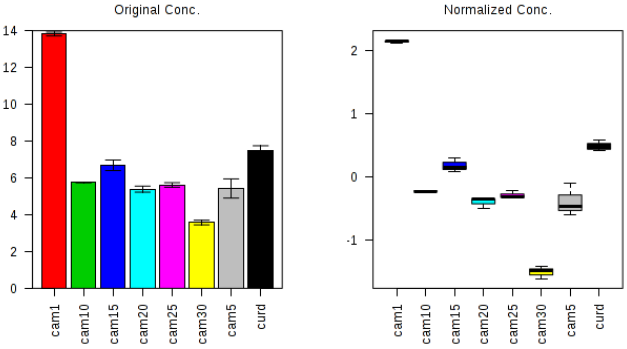
Aspartic acid



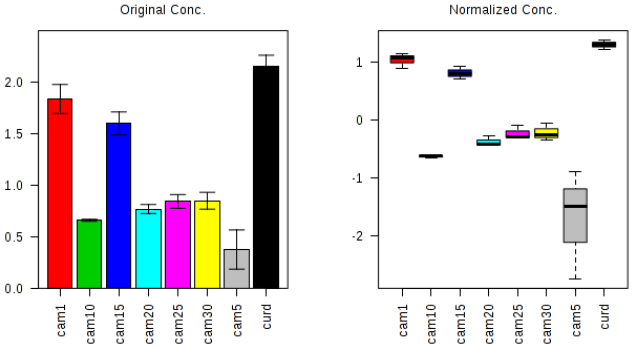
glutamic acid



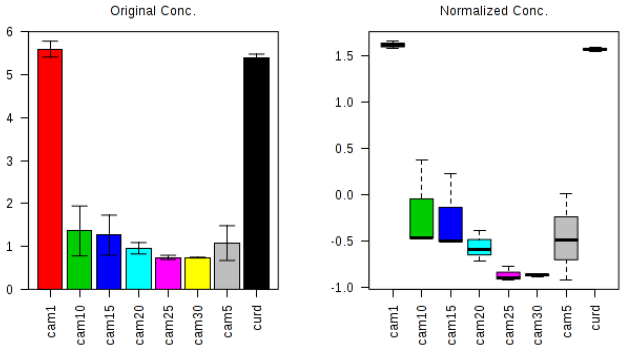
isoleucine



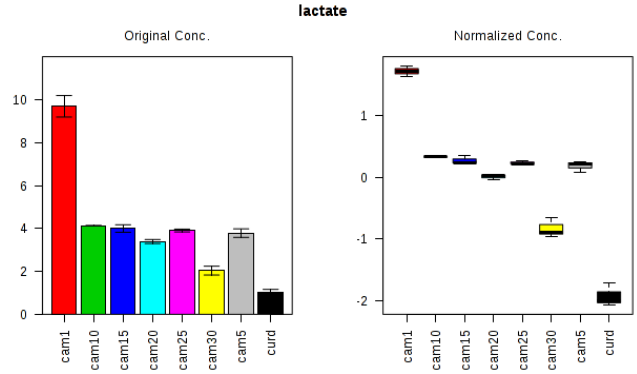
glutamine



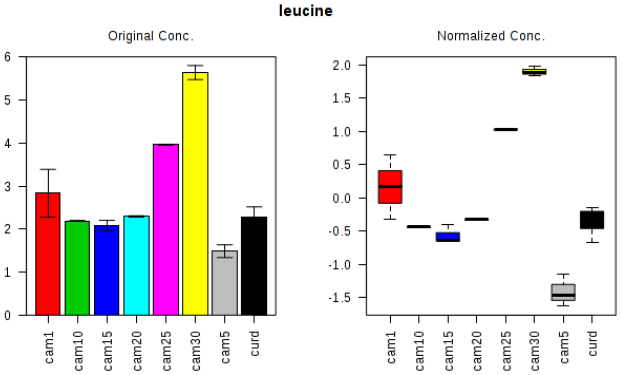
Glycine



lactate



leucine



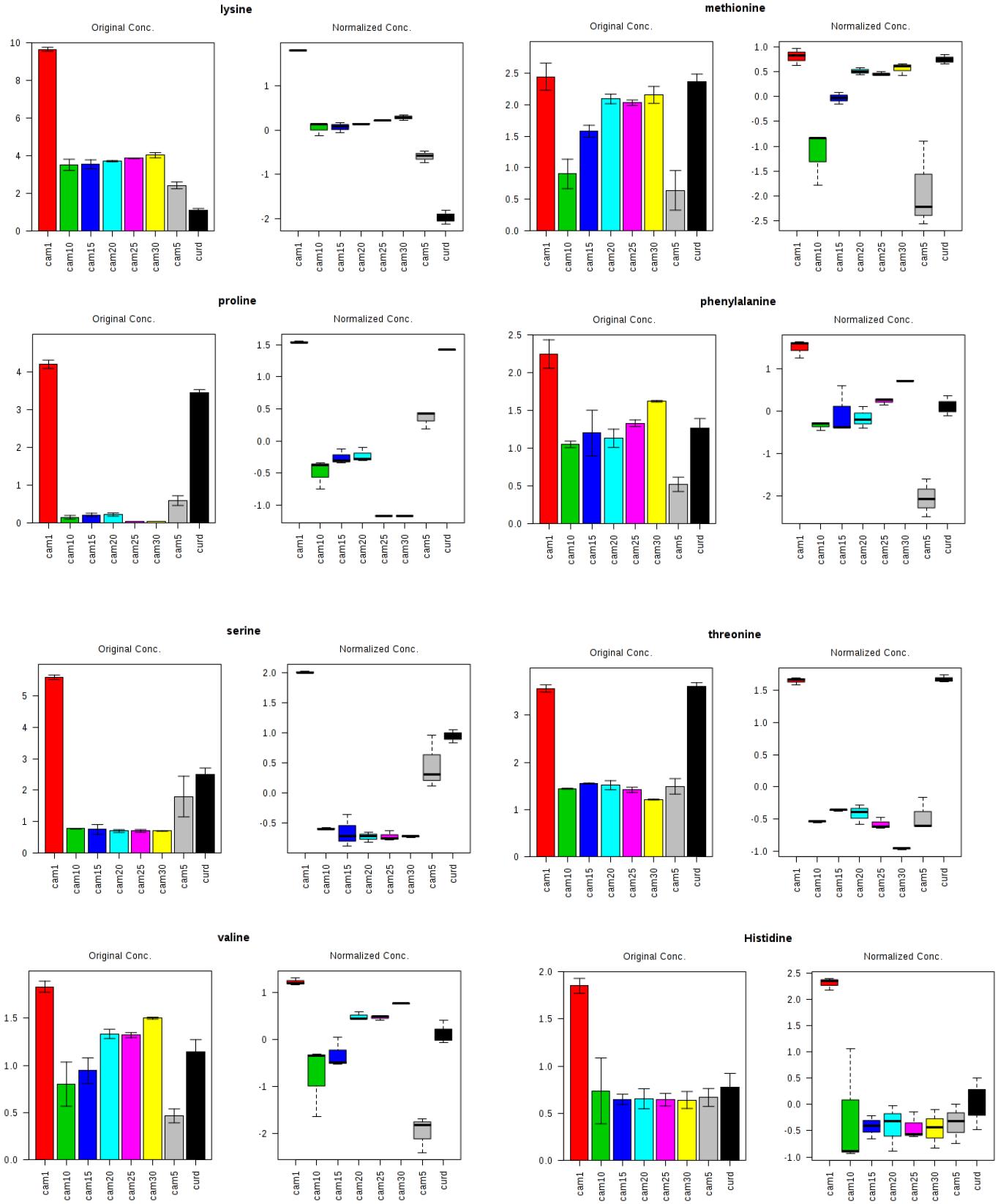


Figure 5-3 Changes in abundances over the ripening time of 15 representative amino acids and also lactate analyzed using ^{1}H NMR. Values are the means of signals abundance of three independent experiments. Error-bars show standard deviation. (The bar plots on the left show the original values (mean +/- SD). The

box and whisker plots on the right summarize the normalized values. Note, positive infinite numbers are represented as 999999, and negative infinite numbers -999999

	Curd	Cam1	Cam 5	Cam 10	Cam 15	Cam 20	Cam 25	Cam 30				
Amino acids	Ave±STV								f.value	p.value	Log 10	FDR
Aspartic acid	2.80±0.27	3.46±0.27	0.86±0.15	0.49±0.11	0.49±0.1	0.39±0.02	0.28±0.07	0.22±0.02	320.82	5.48E-16	15.261	5.53 E-15
Aspargine	0.63±0.12	2.49±0.22	1.14±0.1	1.05±0.01	1.72±0.29	2.29±0.1	3.61±0.08	2±0.01	311.56	6.92E-16	15.16	5.53 E-15
Glutamic acid	3.15±0.48	3.55±0.13	0.54±0.8	2.43±0.23	2.49±0.44	2.19±0.06	3.38±0.06	3.57±0.01	260.1	2.89E-15	14.539	1.52 E-14
Glycine	5.38±0.09	5.59±0.19	1.07±0.4	1.36±0.58	1.26±0.46	0.95±0.13	0.74±0.04	0.74±0.01	244.28	4.74E-15	14.324	1.52 E-14
Phenylalanine	1.26±0.12	2.25±0.19	0.52±0.09	1.05±0.04	1.2±0.3	1.13±0.12	1.33±0.04	1.62±0.01	244.21	4.75E-15	14.323	1.52 E-14
Valine	1.14±0.12	1.83±0.06	0.46±0.07	0.8±0.23	0.94±0.13	1.33±0.04	1.32±0.02	1.5±0.01	112.5	2.10E-12	11.678	5.60 E-12
Threonine	3.60±0.08	3.57±0.08	1.49±0.16	1.44±0.01	1.55±0.01	1.51±0.09	1.41±0.05	1.21±0.01	102.52	4.33E-12	11.364	9.89 E-12
Serine	2.49±0.20	5.59±0.07	1.79±0.64	0.77±0.01	0.74±0.15	0.69±0.04	0.7±0.04	0.7±0.01	86.334	1.64E-11	10.785	3.28 E-11
Proline	3.45±0.07	4.2±0.11	0.59±0.13	0.14±0.04	0.21±0.04	0.22±0.04	0	0	82.729	2.28E-11	10.642	4.06 E-11
Methionine	2.36±0.12	2.44±0.22	0.64±0.31	0.9±0.23	1.57±0.09	2.09±0.07	2.03±0.04	2.15±0.13	64.006	1.64E-10	9.7848	2.63 E-10
lysine	1.09±0.10	9.66±0.11	2.42±0.18	3.51±0.29	3.54±0.23	3.7±0.03	3.86±0.01	4.02±0.13	37.266	9.68E-09	8.0139	1.41 E-08
Leucine	2.27±0.24	2.83±0.56	1.48±0.14	2.19±0.01	2.08±0.11	2.29±0.01	3.96±0.01	5.63±0.16	34.704	1.64E-08	7.7853	2.19 E-08
Histidine	0.77±0.14	1.85±0.08	0.66±0.09	0.73±0.34	0.64±0.05	0.65±0.1	0.64±0.6	0.64±0.09	27.086	9.99E-08	7.0005	1.23 E-07
Isoleucine	7.49±0.25	13.82±0.11	5.42±0.52	5.75±0.01	6.68±0.28	5.39±0.16	5.61±0.12	3.57±0.12	24.533	2.03E-07	6.6923	2.32 E-07
Glutamine	2.15±0.10	1.84±0.14	0.37±0.19	0.66±0.01	1.6±0.11	0.77±0.04	0.84±0.06	0.85±0.08	20.679	6.79E-07	6.1684	7.24 E-07
Lactate	1.04±0.12	9.69±0.5	3.78±0.19	4.13±0.01	3.99±0.17	3.39±0.09	3.9±0.06	2.03±0.2	10.149	7.42E-05	4.1298	7.42 E-05

Table 5-1 metabolites identified for Camembert cheese from ^{1}H RMS fingerprints that exhibited significant changes in concentration during 30 days of ripening. (Values of the Average±STDEV are for three independent experiments. Different superscripts indicate significant differences ($p<0.05$)

The increased levels of several amino acids in the most ripened cheese samples, including Aspargine, leucine, are consistent with a successful proteolysis which is one of the major processes in the formation of the texture, flavour and aroma during cheese maturation (Fox and McSweeney, 2004). It was also observed that some of free amino acids such as glutamic acid and aspartic acid were degraded during maturation. Piras et al. (2013) described how a decreased amount of glutamic acid and an increased content of γ-aminobutyric acid (GABA) at the late stages of maturation were observed to be a concomitant phenomenon in chedder. The relationship between these two metabolites was believed due to the ability of some mesophilic lactobacilli to produce GABA by decarboxylation of glutamic acid. In the past, GABA was noted in mature cheeses as an indication of anomalous fermentations producing organoleptically unfavourable cheeses (Piras et al., 2013) but here it seems to be a normal part of the maturation process. This indicates that data for the maturation of hard cheeses may not be the same as for soft cheese.

Hierarchical Cluster Analysis (HCA) was applied on each sample. The data show that the proteolytic process is substantial the first five days of ripening. The results clearly show an increase in total amount of amino acids between curds to day 1, while a decrease after day 1 to day 5 (Figure 5.5). This confirms the results obtained with deferent techniques (Addeo et al., 1995; Cattaneo et al., 2008).

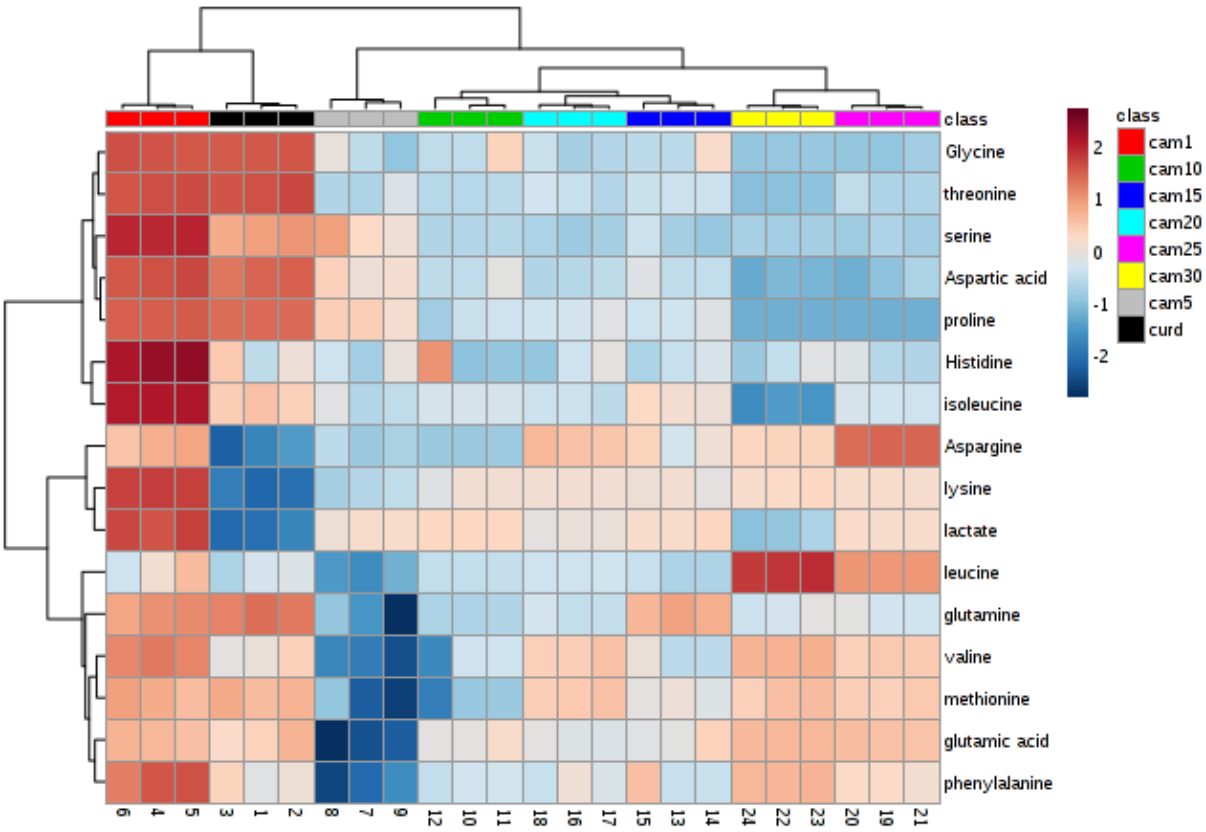


Figure 5-4 Hierarchically clustered heat map of the different amino acids in Camembert cheese samples during 30 days of ripening. Row represents different cheese samples and column amino acids concentration. Cells are coloured based on amino acids concentration in cheese samples. Red represents high concentration and blue showed low concentration in cheese samples during ripening

5.5.1 PCA and PLS-DA

Multivariate analyses were employed to analyse the NMR metabolic profiles and also to identity any underlying variability that could be related to metabolic differences in Camembert cheeses. The full ^{1}H NMR spectra obtained for all of the cheese samples were used to obtain a data matrix; PCA of all profiles did not identified any samples as outliers, and visual inspection of the spectra confirmed this.

The performance of the PLS-DA models were evaluated by using cross validations (CV) with increasing numbers of components created using the specified number of variables. The first and second components are shown in PLS-DA and VIP scores plot for Camembert cheese samples at different time of ripening in Figure 5.6 A and B, which has interpretation of the metabolic profiles of amino acids by maturation time. Ripening dominates the profiles as seen by different coloured symbols in Figure 5.6 A. Observation of the loadings for Figure 5.6 again confirms that protein proteolysis increases during ripening.

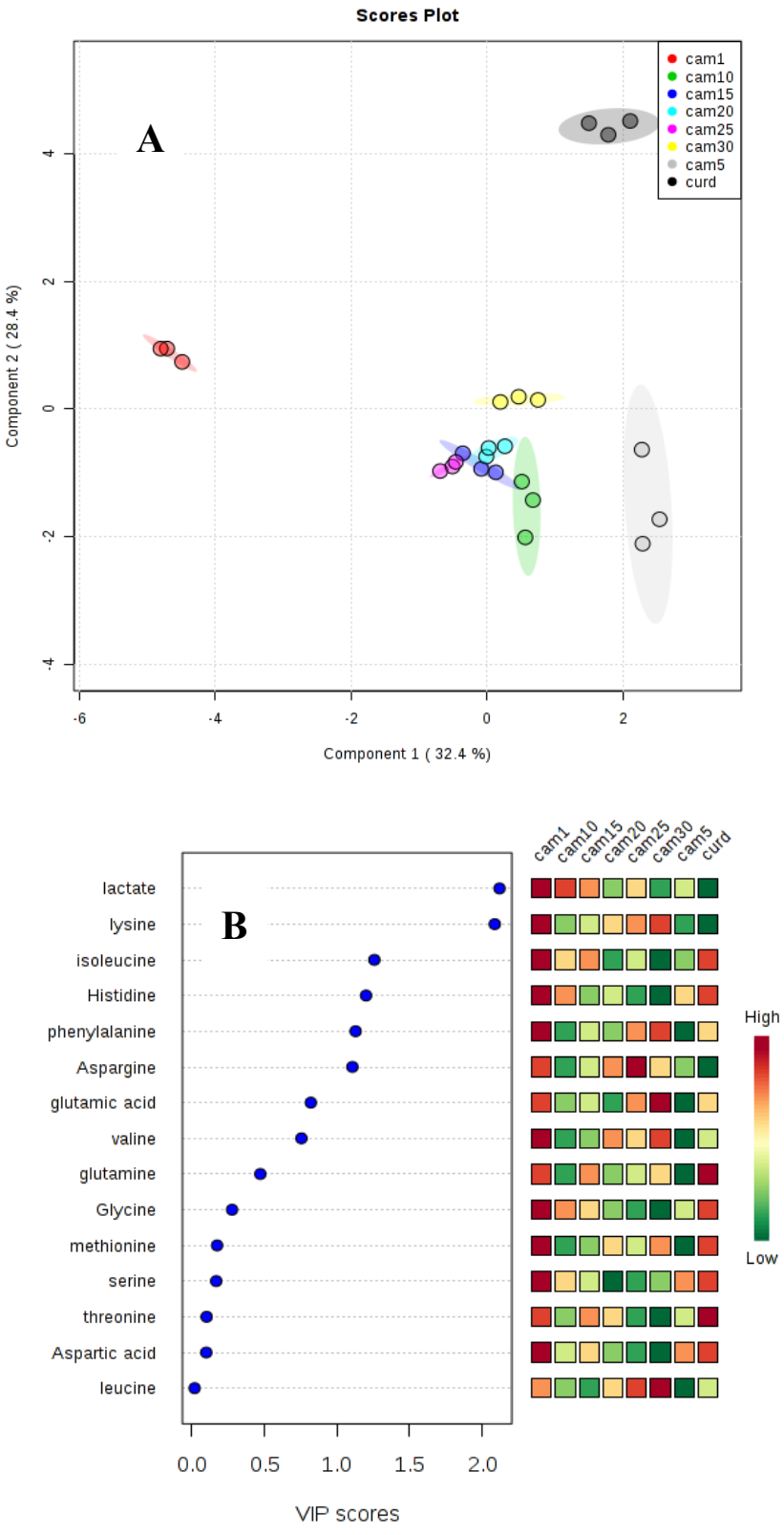


Figure 5-5 PLS-DA (A) and VIP (B) scores for Camembert cheese samples at different time of ripening

Four PC's adequately explained 96.2% of the variance, with PC1 (44.4%), PC2 (29.6%) explaining the majority of this. The 2D scores plot in Figure 5.7 A showed that clear separation

among samples according to different ripening stage is feasible. The corresponding "Biplot" Figure 5.7 B clearly specified samples with different ripening stage were characterized by different amount of amino acids. In particular younger samples (Curd and day 1) were characterized by larger content of threonine (at 1.31–1.33 ppm) and glycine (from 3.53 to 3.57 ppm), serine (at 3.83–3.85 ppm and from 3.93 to 3.99 ppm), isoleucine (at 0.99–1.01 ppm), lactate (from 1.33 to 1.35 ppm and from 4.09 to 4.17 ppm), lysine (from 1.39 to 1.53 ppm, from 1.67 to 1.73 ppm, from 1.85 to 1.91 ppm, from 2.99 to 3.03 ppm and from 3.73 to 3.77 ppm), proline (from 1.97 to 2.07 ppm, from 2.31 to 2.39 ppm and from 4.09 to 4.15 ppm),; medium aged samples (5 to 15 day) were characterized by lower amounts of amino acids, like, isoleucine (at 0.99–1.01 ppm) and glutamic acid (from 2.01 to 2.15 ppm, from 2.31 to 2.39 ppm and at 3.73–3.75 ppm),; older samples (20 to 30 day) were characterized by larger content of aspargine (at about 2.93 ppm) and leucine (at 0.95–0.97 ppm). These changes in amino acids content is in according to previous results (Angelis et al., 2000) confirms the significant variation, gives an index of the proteolytic process as expected in ripening. The small amount of isoleucine in aged camembert samples resulted in agreement with the important catabolism of the branched amino acids, where isoleucine and valine were degraded by a number of pathways into volatile compounds, important for cheese flavour (Consonni and Cagliani, 2008). Abbreviations for three independent experiments characterising sample distribution are shown in Table 5.2 and link to figure 5.7.

Days	Samples
Curd	1,2,3
Cam 1	4,5,6
Cam 5	7,8,9
Cam 10	10,11,12
Cam 15	13,14,15
Cam 20	16,17,18
Cam 25	19,20,21
Cam 30	22,23,24

Table 5-2 Abbreviations for three independent experiments characterising sample distribution

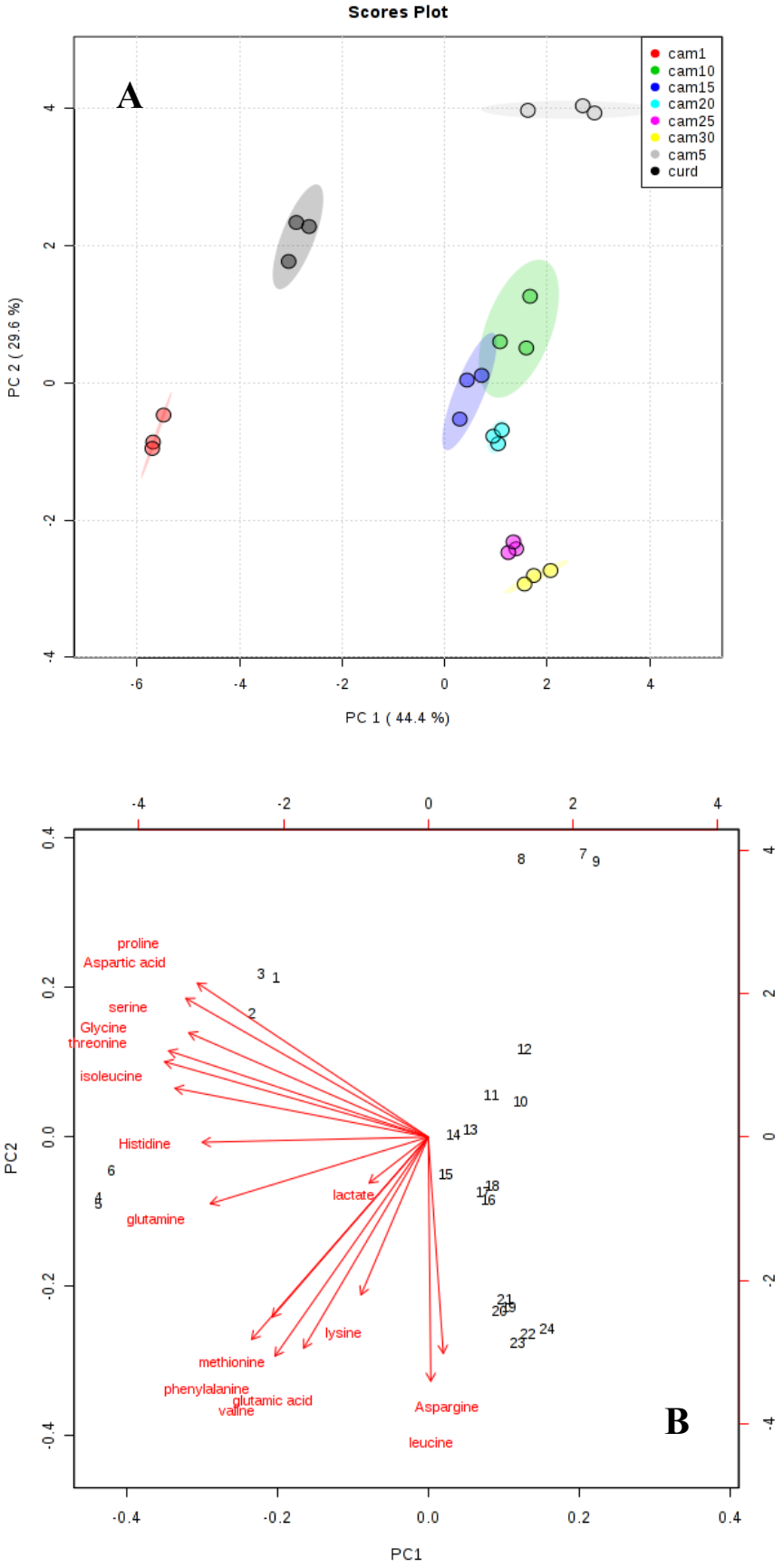


Figure 5-6 PCA of ^{1}H NMR data. (A) Score plot and (B) Loading plot where metabolites are reported. Abbreviations as in Table 5.2. Taken together the two plots allows for the identification of metabolites responsible for separation of the samples.

Amino acids	VIP	Coefficient values	Curd	Cam1	Cam5	Cam10	Cam15	Cam20	Cam25	Cam30
Aspartic acid	0.100	16.112	7.6365	34.045	27.982	9.0154	3.572	7.787	19.805	19.051
Aspargine	1.107	23.396	57.436	17.042	44.31	4.7427	0.20333	5.048	27.84	30.55
glutamic acid	0.820	23.696	43.546	27.614	43.591	7.4873	1.8316	7.1229	28.409	29.962
Glycine	0.278	16.085	21.762	40.147	20.05	9.0714	3.7632	6.8505	12.849	14.191
phenylalanine	1.129	19.485	16.708	27.401	39.214	8.1729	2.6166	7.8935	27.336	26.539
valine	0.756	20.425	21.728	24.787	41.997	7.6463	2.1833	7.6442	28.982	28.435
Threonine	0.102	16.138	23.749	41.983	18.553	9.2545	3.898	6.8028	11.589	13.271
Serine	0.169	17.164	24.387	38.256	23.595	8.8589	3.4925	6.9248	15.244	16.553
Proline	0.018	16.727	8.0559	32.678	30.504	8.9188	3.4224	7.9115	21.626	20.702
Methionine	0.175	22.73	44.685	31.31	38.199	7.7749	2.1756	6.8591	24.355	26.484
Lysine	2.087	26.586	83.66	31.152	37.913	6.1281	0.93415	4.7958	21.038	27.066
Leucine	0.02	19.017	28.01	7.0177	45.76	3.9418	0	5.3725	31.262	30.769
Histidine	1.201	15.165	21.762	40.147	20.05	9.0714	3.7632	6.8505	12.849	14.191
Isoleucine	1.258	17.084	38.83	44.52	15.308	8.9066	3.6893	5.9471	8.0012	11.468
Glutamine	0.472	18.339	45.224	40.669	19.322	8.1646	3.0818	5.6199	10.443	14.185
Lactate	2.120	23.053	100	35.126	19.771	4.7942	0.74016	2.0996	6.4092	15.481

Table 5-3 Metabolites with their influence on projection (VIP) and their corresponding coefficients for each Camembert samples at different time, as calculated by PLS-DA. Higher coefficient values indicate higher comparative levels of the corresponding metabolite in different cheese samples

All the metabolites having a VIP score were considered and reported in Table 5.3, together with their corresponding coefficient values for each sample of Camembert. In order to evaluate the importance of each amino acid the VIP (Variable Importance in the Projection) calculated for all parameters. Compounds with largest VIP were the most relevant for explaining the response. From the Figure 5.6 B it was evident that the most affecting variables were lactate (which is not an amino acid) and lysine, followed by other amino acids like aspargine,

phenylalanine, histidine and isoleucine. According to these results, concentrations of amino acids that had a high correlation coefficient with age of cheese can be used as an index of ripening. A similar study has been made of Cheddar cheese (hard cheese) using HPLC profiles of water extracts; in that study, samples were classified into mild, medium, old, and extra old (Pham and Nakai, 1984).

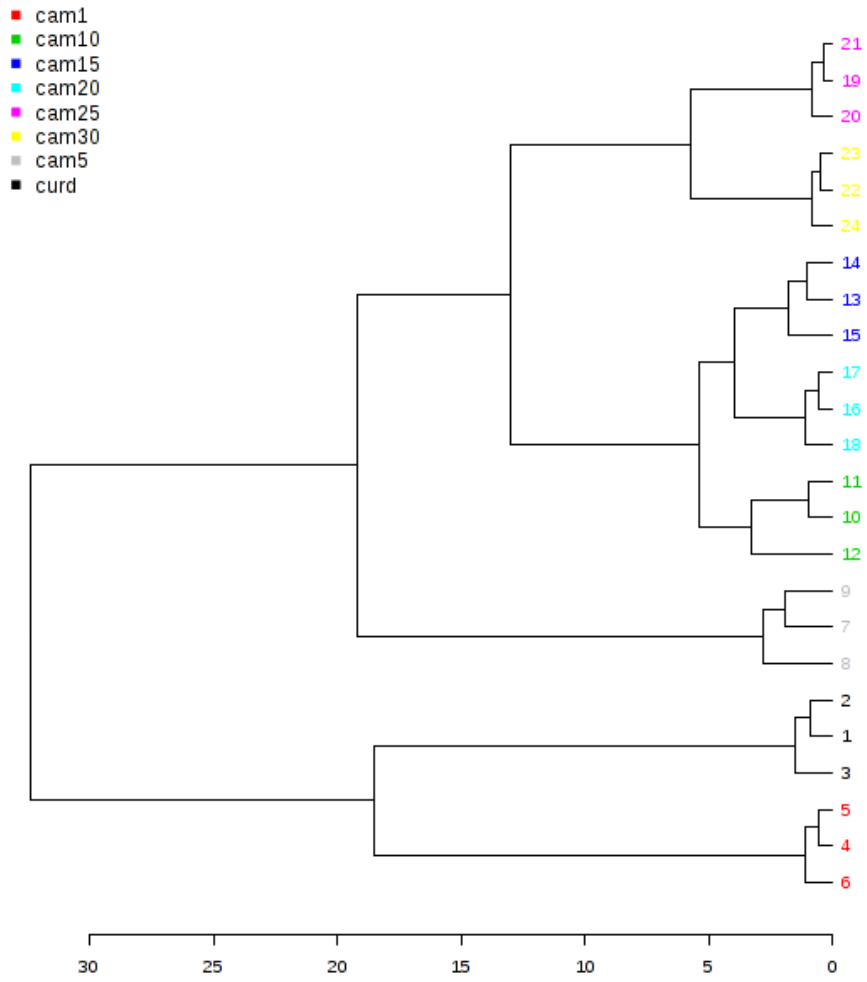


Figure 5-7 Dendrogram corresponding to the cluster analysis for amino acid concentrations in Camembert cheese during ripening. Samples are grouped according to the similarity of correlation coefficients between concentrations versus age. Scale represents the distance between the groups

Figure 5.8 is a dendrogram for amino acid concentrations in Camembert cheese during ripening. From this dendrogram eight groups of Camembert cheese samples were analysed. Cluster analysis grouped Camembert samples with similar ripening patterns but did not

establish the grouping criteria. In contrast The metabolomes of the eight samples resulted clearly different by HCA, where the related statistical dispersions showed that the different patterns of appearance of amino acids could be caused by their different uses by the microorganisms that were present in the curd. During ripening, some amino acids are used as a substrate for the formation of biogenic amines and other compounds (García-Palmer et al., 1997). The main differences appeared from curd and day 1 of ripening. The medium aged was the point at which the pattern of appearance of free amino acids changed.

5.6 Conclusion

The present work demonstrated the use of ^{1}H NMR metabolomics for investigating the ripening process of camembert cheese based on amino acid content.

The use of multivariate data analysis (chemometrics) in combination with ^{1}H NMR spectroscopy data proved to be an efficient tool for metabolite fingerprinting of soft cheeses.

The results proved that ^{1}H NMR metabolomics can provide a rapid and unique technique to evaluate the fermentative process during cheese manufacturing, which has ability to provide better understanding of how to control cheese quality. ^{1}H NMR and statistical approach confirmed to be a valid tool in food analysis, as already highlighted in the literature. This is the first time this method has been used for a soft cheese.

This method can be applied successfully for ripening differentiation of camembert cheese with good reliability. This method revealed the capability to obtain samples discrimination, providing a reliable analytical method. This procedure could complement the traditional systems of quality control and could have significant impact on industry.

Chapter 6 PREDICTIVE MODEL OF CAMEMBERT CHEESE RIPENING

6.1 Introduction

Modelling the process of cheese ripening remains a challenge as it is a complex system. There is still lack of knowledge to understand the interactions that take place during the process at different scale levels. However, knowledge can be gathered from the skills of scientific and operational experts.

The maturation of camembert cheese is a complex system with numerous interacting variables responsible for physical, chemical, biological and structural changes. As in many food processes, both automatic monitoring and human operators are require to monitor the process of cheese maturation in order to maintain product quality (Ilyukhin et al., 2001). Quality depends on environmental factors such as relative humidity, temperature and gas concentration in the chamber, and the interactions between inoculated micro-organisms and curd substrates resulting from variations in the quality of raw milk and conditions of cheese making.

In order to understand this process from a microbial, physicochemical, biochemical or sensory point of view, many studies have been carried out. Authors such as Leclercq-Perlat et al. (2004) studied interactions between microflora in order to assess their synergistic effects on substrate consumption. Instrumental methods were also developed to provide an objective method for characterising organic and physicochemical cheese characterisation during the maturation process of cheese (Pérès et al., 2002; Sicard et al., 2011a). Hébert et al. (1999) studied sensory properties to find relationships between cheese organoleptic properties and microbiological, biochemical and physicochemical parameters modifications. Some authors have tried to model part of the process more recently. During the aging process Helias et al. (2007) set up a dynamic model of camembert mass loss resulting from physical and biological phenomena. However, the prediction of microflora growth has not been integrated into the model and there is still a lack of knowledge. To understand the numerous variables and their interactions, large databases are required. Due to time limits, financial constraints and scientific and technological obstacles, these databases are generally non-existent. The cheese maker usually controls the aging process in factories by means of a limited number of instrumental measurements and empirical sensory perception (Sicard et al., 2012). However, they are able to manage the

complexity of the process and play a decisive role in their evaluation and reasoning (Sicard et al., 2012). For example Perrot et al. (2004), successfully built a decision support system to control the maturation of a soft mould cheese based on expert skills. The impact of the results was limited due to the lack of embedded microbial kinetics. However, this knowledge can be extracted from the knowledge of cheese scientists working in the field of dairy science, such as microbial kinetics.

Sicard et al. (2011b) showed that expert cheese-makers distinguished 4 main chronological steps in Camembert ripening in their study. The results are presented in a new ways in Figure 6.1. In the first step, the Camembert type cheese has a high surface humidity, white colour, and a fresh or lactic odour. The cheese becomes less moist in the second step, the colour gradually from changes from white to creamy yellow, and the cheese has a white *P. camemberti* coat, and a dominant mushroom odour is evident. In step 3, the *P. camemberti* coat completely covers the cheese, the under-rind begins to grow and the typical Camembert odour appears. Finally, in step 4, the cheese is almost at the end of the optimum consumption period with a strong ammonia odour, the thickness of the under-rind is complete and the colour of the rind becomes darker under the *P. camemberti* coat. Expert knowledge is obviously not limited to these 4 steps, but it represents the major steps of cheese evolution during maturation time. However, it can help cheese industries to assess the whole ripening process.

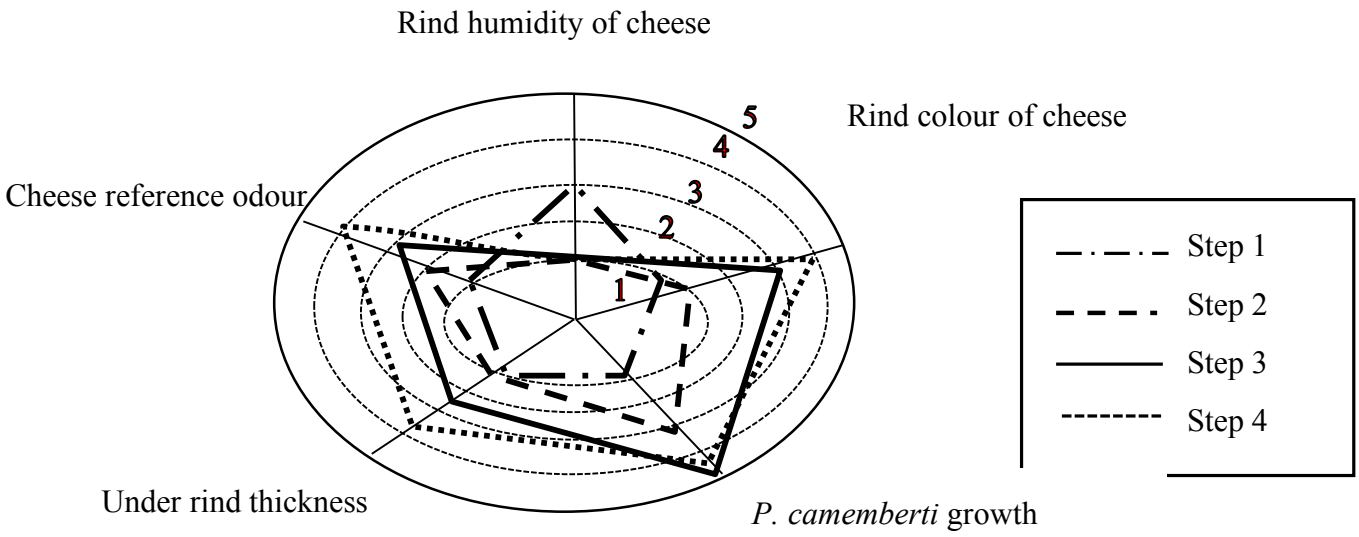


Figure 6-1 Evolution of cheese sensory profile throughout ripening adopted from Sicard et al. (2011b)

In recent years, multivariate statistical techniques have been used to analyse, describe and interpret multidimensional observations in general. PLS regression is a multivariate statistical technique used in food research to obtain calibration models as an alternative to other statistical techniques, such as multiple linear regression (MLR) and principal component regression (PCR) (Molina and Martın-Alvarez, 1996; Pripp, 1999; Pripp et al., 2000). PLS regression has been applied to various cheese varieties in dairy products, to identify quantitative relationships between sensory attributes and certain chemical variables. Based on the volatile composition, Barbieri et al. (1994) used partial least squares (PLS) regression to predict parmesan cheese flavour. In Cheddar cheese, Frister et al. (2000) used the same statistical approach to correlate chemical parameters such as peptides, caseins and casein degradation products, with the bitter taste of cheese.

Due to their high moisture content and the rapid growth of surface mould, soft cheeses like Camembert mature very quickly. In addition to the proteolytic actions of the coagulant and the protease from the starter culture, the action of the mold protease, transforms the insoluble casein into fragments of acid-soluble nitrogen (ASN). In order to produce the distinctive bloomy, edible rind and creamy interior texture characteristic of Camembert cheese, it takes 3 weeks of maturation before marketing (Smith, 2005). Once the cheeses are mature enough, they are wrapped in paper and can be placed for transportation in wooden boxes. However, the Camembert cheeses manufacturing date is not required on the marketplace. That means that Camembert cheeses available in the market have different sensory properties, so for marketing purposes a model for predicting the ripening time for Camembert cheese as was demonstrated in this thesis could be of a great interest. To calculate the ripening time of commercial cheeses based on physicochemical and proteolysis parameters Ruiz et al. (1998) applied various multivariate regression techniques, namely MLR, PCR and PLS. These authors found that the PLS regression yielded the best predictions of cheese maturation time. The results however, were preliminary and it was concluded that it would be necessary to check the method's predictive capacity and use a larger number of cheese samples to draw a more statistically based conclusion. This check has now been undertaken as part of this thesis.

Gan (2015), showed physical measurements proved not important in predicting age of different cheeses. Furthermore, food colours have been commonly added in many of the industrial varieties of cheeses, which make the measurement less distinguishing.

The aim of this work was to build PLS regression model to interpret and predict the age of Camembert derived from GC-MS-SPME, ^{1}H NMR and preliminary tests to replace the expensive and time-consuming sensory panels and cheese graders. This is a modification to understand the soft modelling approach based on chemometric analyses of the chromatographic profiles of a real food with extensive commercial diversity, cheese volatile compounds during different ripening stage.

PLS combines the properties of multiple linear regression and PCA to make linear combinations in the dependent matrix. By use of these regression models, relationships between amino acids, aroma compounds, total solid, water activity, moisture content and pH and maturity of Camembert cheese were established. Cross-validation was used in order to optimize the use of a small set of measurements.

6.2 Material and methods

6.2.1 Camembert cheese samples

Cheese samples were also sourced from LD&D Foods Pty Ltd. These cheeses were ripened at the fridge (2-4°C) for 30 days. Samples taken at deferent stages of ripening (Cam day 1, Cam day 5, Cam day 10, Cam day 15, Cam day 20, Cam day 25 and Cam day 30). The samples were placed in special containers and kept in liquid nitrogen until used in order to maintain their characteristics and avoid degradation.

6.2.2 Analytical methods

6.2.2.1 Compositional analysis

Total solid (Ts), pH, water activity (a_w) and moisture content (MC) were determined as described in chapter 4.

6.2.3 Assessment of flavour compounds and proteolysis

Data from chapter 3 and 6 were used to assess flavour compounds and amino acid metabolism, respectively.

6.2.4 Statistical method

PRIMER software was used to assess the link between physico-chemical properties and chemometrics data (GC-MS-SPME and ^{1}H NMR), which described by Clarke and Ainsworth (1993). Parameters correlated with Spearman rank correlation method.

The data from each replicate were analysed statistically by using the Minitab 18.1 (Minitab Inc., US). The partial least square regression (PLS) performed with full cross validation, used to analyse the aroma and amino acids profile and predict the maturity of the camembert cheese base on these compounds. Removing groups of samples in the modelling and testing the performance of the model using the remaining data set is an evaluation tool to check calibration model which called full cross validation.

All variables were auto scaled before chemometrics application so the effect of drifts and baseline were removed. PLS regression was applied to determine the relationship between predictor variables (such as the total solid, water activity, and moisture content and pH, and GC-MS-SPME and ^{1}H NMR data) and the ripening stage of camembert cheese. Besides, PLS was used as an exploratory analysis tool to select suitable predictor variables to predict linear model. PLS was applied to model the ripening and maturity of camembert cheese using the chemometrics data with predictive ability.

6.3 Results and discussion

6.3.1 Correlation of physico-chemical properties and chemometrics data using PRIMER

Physio-chemical properties of camembert cheeses including pH, water activity, moisture content and total solid were measured for all samples (see chapter 4 for details). A PCA plot of physico-chemical data for both ^{1}H NMR and GC-MS data sets (Figure 6.2 and 6.3) showed a clear separation between Camembert cheese samples during ripening. Step-wise selection distance-based linear modelling (DistLM) using the physico-chemical dataset available for

both ^{1}H NMR and GC-MS allowed us to determine the relative contribution of physico-chemical variables in explaining separation in Camembert cheese samples during maturation.

To explore whether there is a significant relationship between physico-chemical and chemometric variable, resemblance matrix from ^{1}H NMR and GC-MS data were overlaid with the resemblance matrix from physico-chemical data (Figure 6-2 and Figure 6-3, respectively). Spearmen rank correlation coefficient of 0.69 and 0.65, shows a significance degree of similarity (R=%1) between ^{1}H NMR and GC-MS matrices with physico-chemical data set respectively (see appendix 1 and 2 for more details).

To explore which set of physico-chemical variable is best explaining the patterns in chemometrics data, Distance based linear modelling (DisTlm) was conducted. Results for ^{1}H NMR data showed all the physico-chemical variables correlated significantly with cheese samples during ripening time, with P-values <0.05 (see Table 6-1). However, in increasing order, total solids and pH together explained the best combination of %57 of total variation during ripening with ^{1}H NMR data.

Marginal tests		Step-wise selection Sequential tests		
Variable	P value	Variable	P value	Cumul.
pH	0.03	+Ts	0.001	0.42
a_w	0.003	+pH	0.001	**0.57**
Ts	0.001			
Mc	0.001			

Table 6-1 DistLM results of ^{1}H NMR data against physico-chemical variables during Camembert cheese ripening

GC-MS data set showed similar results for all the physico-chemical variables which correlated significantly with cheese samples during ripening time, with P-values of <0.05. Furthermore,

in increasing order for GC-MS data set, pH, total solids and moisture content together explained %76 of combination of variation (see Table 6.2).

In this study, both DistLM's (Table 6-1 and 6-2) suggest that a_w were not as influential as other physico-chemical variables. This may be attributed to the fact that as proteolysis increased; the hydrophilic groups resulting from proteolysis held or stabilized the maximum amount of water (Schlesser et al., 1992).

Marginal tests		Step-wise selection Sequential tests		
Variable	P value	Variable	P value	Cumul.
pH	0.034	+Mc	0.001	0.46
a_w	0.001	+pH	0.001	0.670
Ts	0.001	+Ts	0.002	**0.76**
Mc	0.001			

Table 6-2 DistLM results of GC-MS data against physico-chemical variables during Camembert cheese ripening

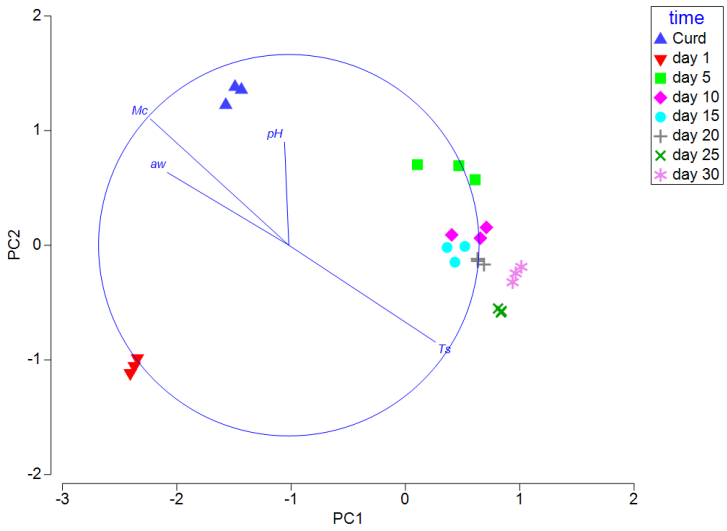


Figure 6-2 PCA analysis of Camembert cheese samples in relation to physico-chemical variables during ripening based on ^{1}H NMR data

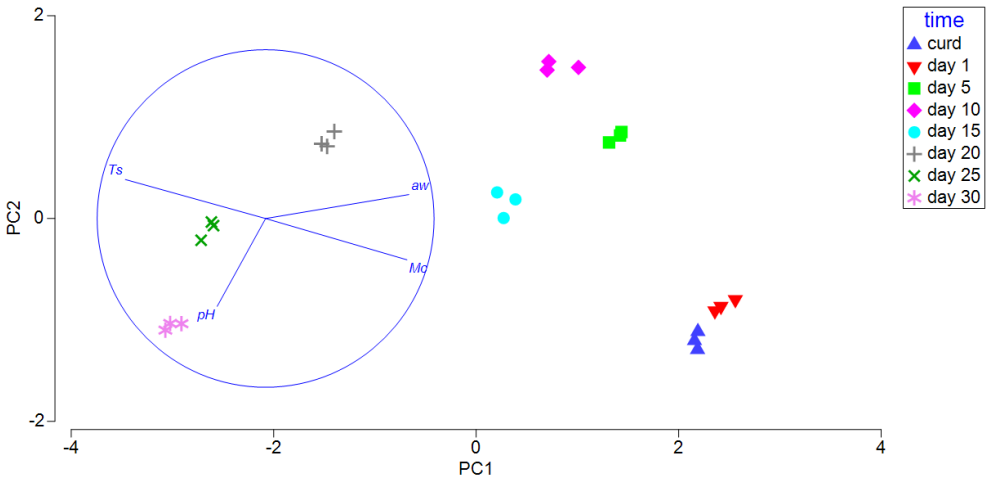


Figure 6-3 PCA analysis of Camembert cheese samples in relation to physico-chemical variables during ripening based on GC-MS data

6.3.2 PLS modelling of Camembert cheese ripening

Table 6.3 shows the means of the analysed variables (a_w, pH, Ts, MC) in Camembert cheeses (n=32). The a_w values decreased significantly ($P<0.05$) during the aging period. However, in contrast, Ts values increased progressively ($P<0.05$) from day 1 to 30 days of ripening. The pH also increased significantly ($P<0.05$) between the beginning and the end of ripening. These results accord with those obtained by other authors for Camembert cheese (Boutrou et al., 1999; Guizani et al., 2002; Michalski et al., 2003; Schlesser et al., 1992) but go into much greater depth and when combined with the metabolic results, show what is going on at the molecular level.

Variables	Ripening time							
	Curd	1	5	10	15	20	25	30
w	0.98	0.94	0.91	0.89	0.86	0.81	0.76	0.59
H	6.7	3.8	4.1	4.2	4.6	4.9	5.2	5.5

's (%)	36.2	40.83	41.6	45.55	51.34	48.7	50	52.3
Mc	48.71	38.28	37.02	36	33.74	32.92	30.87	29.91

Table 6-3 Mean values (n=32) of the variables analysed in the group of the 96 (24*4) Camembert cheeses, at different ripening times

PLS regression was applied to the 96 samples using the variables showed in Table 6.3. The PLS coefficient plot (Figure 6.4) obtained for 4 variables showed that the most important variables that contributed to predict the ripening time of these Camembert cheeses were pH and a_w. With the aim of reducing the numbers of variables that take part in the prediction model, these two parameters, besides their squares, were considered as predictor variables in the model (see appendix 3 for more details).

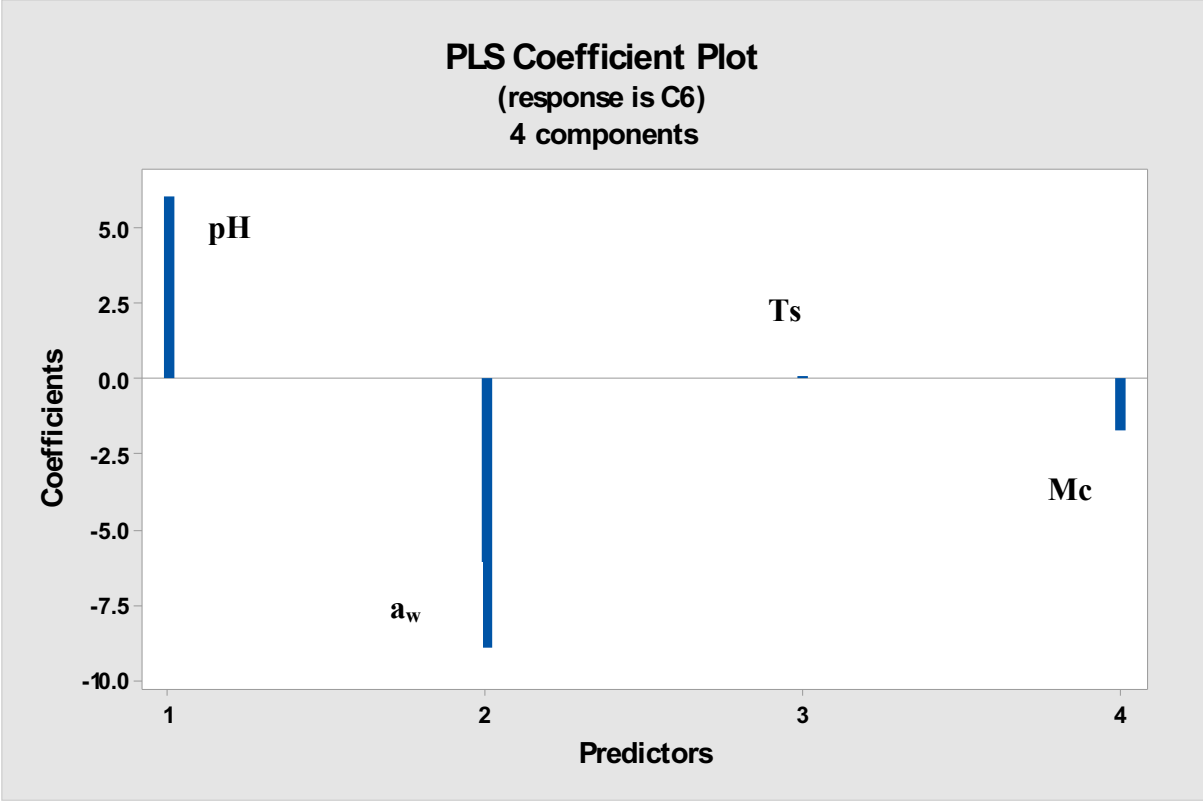


Figure 6-4 PLS Coefficient Plot of physico chemical properties (pH, a_w, Ts and Mc) of Camembert cheese during ripening

The correlation coefficient of the flavour compounds from GC-MS/SPME and time (Figure 6.5) in Camembert cheese samples among 46 flavour compounds indicated 14 compounds with R^2 between 0.70 to 0.95, however, the flavour compounds that were found most important with R^2 greater than 0.90, Hexanoic acid (blue cheese flavour note), Pentan-2-one (fruity flavour note), Heptan-2-one (cheesy flavour note) and Heptan-2-ol (mild ether flavour note) were selected as references for PLS predictive equation. Table 6.4 presents correlation coefficient of the selected compounds (more data in appendix 4), shows the correlation of time and all the flavour compounds driven from GC-MS/SPME).

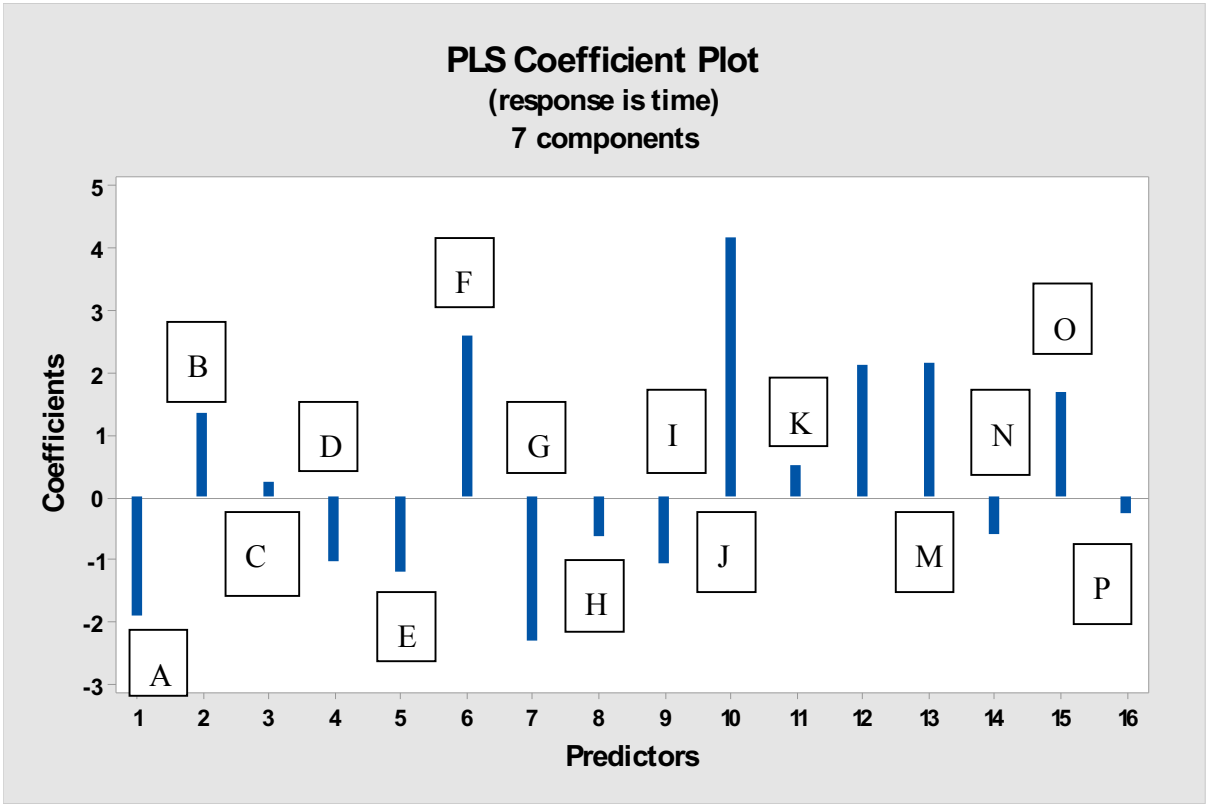


Figure 6-5 PLS Coefficient Plot of Camembert cheese flavour compounds derived from GC-MS/SPME during ripening

Flavour compounds	Root mean square	Abbreviations
Acetic acid	0.78	A
Butyric acid	0.80	B
Hexanoic acid	0.90	C
Decanoic acid	0.84	D
Pentan-2-one	0.93	E
Hexan-2-one	0.83	F
Octan-2-one	0.78	G
4-Heptanone	0.81	H
Heptan-2-one	0.92	I
Heptan-2-ol	0.95	J
Methyl Butanol	0.72	K
Decalactone	0.74	L
Ethyl Butanoate	0.76	M
Butyl Butanoate	0.71	N

Table 6-4 correlation coefficient of the selected compounds

In order to find out the most important amino acids, I calculated the VIP (Variable Importance in the Projection) parameter in chapter 5. VIP plot together with their corresponding coefficient values for each amino acid, it was evident that the most affecting variables were isoleucine and lysine which were selected as references for PLS predictive equation.

The Regression equation obtained was the following:

$$\begin{aligned} \text{Time} = {} & 18.3 + 1.31\ \text{Hexanoic acid} + 0.387\ \text{Pentan-2-one} + 0.244\ \text{Heptan-2-one} \\ & + 14.82\ \text{Heptan-2-ol} - 0.370\ \text{Lysine} - 1.62\ \text{Isoleucine} - 1.46\ \text{pH} - 4.1\ \text{aw} \end{aligned}$$

with eight components selected by cross-validation, R^2 (determination coefficient) = 0.967 and s (residual standard deviation) = 2.38 days. Figure 6.6 represents the fitting plot between the predicted values, obtained by cross-validation, and the real ripening times of Camembert

cheese samples. The prediction of the ripening times of the Camembert cheeses by the PLS model can be considered good. Table 6.5 shows Predictive variables values and predicted ripening times for the 24 Camembert cheese samples. This means I was able to meet the main aim of my thesis as presented in chapter one.

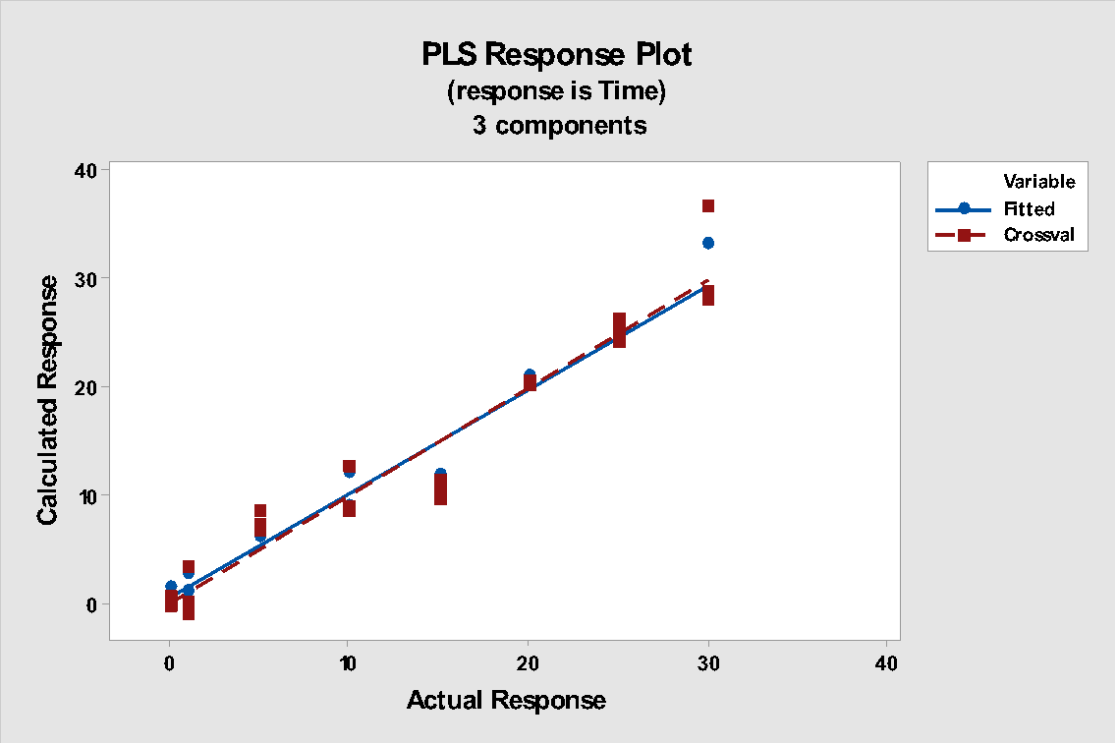


Figure 6-6 Fitting plot between the predicted values, obtained by cross-validation, and the real ripening times of Camembert cheese samples

Sample	Real time (days)	aw	pH	Isoleucine	Lysine	Heptan-2-ol	Heptan-2-one	Pentan-2-one	Hexanoic acid	Predicted time (days)
1	0	0.98	6.7	7.77	1	0	1.0158	0	0	0.9517
2	0	0.98	6.8	7.27	1.08	0	1.2	0	0	0.6605
3	0	0.98	6.6	7.43	1.2	0	1.11	0	0	1.4741
4	1	0.94	3.8	13.82	9.75	0	1.2296	0	0.2595	1.1666
5	1	0.97	4.0	13.92	9.68	0	1.8	0	0.32	0.2073
6	1	0.85	3.6	13.71	9.54	0	2	0	0.277	2.8026
7	5	0.91	4.1	5.01	2.43	0	1.1133	0.6549	0.2575	6.805
8	5	0.94	4.3	6.01	2.24	0	0.98	0.682	0.231	6.2493
9	5	0.82	3.9	5.26	2.6	0	1.4	0.76	0.289	7.2293
10	10	0.89	4.2	5.75	3.68	0.2856	2.5392	0.678	0.3359	8.6345
11	10	0.92	4.4	5.76	3.69	0.3	2.2	1.97	0.36	8.9631
12	10	0.8	4	5.74	3.17	0.45	2.8	1.54	0.37	12.0124
13	15	0.86	4.6	6.62	3.75	0.3551	5.3255	2.2596	0.4891	10.7735
14	15	0.89	4.8	6.44	3.29	0.42	5.4	3.45	0.51	11.9336
15	15	0.77	4.4	7	3.59	0.25	5.8	2.28	0.47	10.212
16	20	0.81	4.9	5.49	3.74	0.4959	14.2142	14.6182	0.6582	20.5053
17	20	0.84	5.1	5.2	3.68	0.58	14	14.73	0.72	21.0884
18	20	0.72	4.7	5.48	3.7	0.4	13.7	14.38	0.81	20.2299
19	25	0.76	5.2	5.55	3.86	0.7177	31.3597	16.1621	0.8683	25.298
20	25	0.79	5.4	5.76	3.87	0.8	32	15.94	1.05	26.0568
21	25	0.67	5	5.54	3.85	0.52	30.8	16.66	1.02	24.2704

22	30	0.59	5.5	3.69	4.04	0.9482	32.6854	17.6768	1.8171	28.7876
23	30	0.62	5.7	3.6	4.15	1	32	18.45	1.66	28.5388
24	30	0.5	5.3	3.44	3.88	1.2	33.1	17.77	2.1	33.1495

Table 6-5 Predictive variables values and predicted ripening times for the 24 Camembert cheese samples

6.4 Conclusions

Ripening time prediction model for Camembert cheese obtained in this study, modified the equation proposed by Ruiz et al. (1998), since it included more variables that take part in the predictive equation (pH, aw, amino acids (lysine and isoleucine) and flavour compounds (hexanoic acid, heptan-2-one, pentan-2-one and Heptan-2-ol). The predicted time of maturation of camembert cheeses is very easy to calculate by these eight parameters which constitute very simple and fast analysis. This new model that combines data and features gathered from multiple approaches has the potential to replace the expensive and time-consuming sensory panels and cheese graders to predict and control cheese maturity and quality.

Chapter 7 DISCUSSION AND CONCLUSION

7.1 General conclusion

This study characterized the flavour development of Camembert cheese during ripening using GC-MS-SPME and ^{1}H NMR to identify biomarkers, developed a calibration model to predict moisture, fat, protein and total solid, and also generated a new a model for Camembert maturity by using experimental and chemometrics data.

Ripening had a marked impact on Camembert cheese's flavour profile of, with more aged Camembert displaying greater intensities of characteristics such as fruity, sweet, vinegar and buttery aromatic. A total of 47 aroma compounds were found to contribute to the overall flavour of Camembert cheese, selected by GC-MS-SPME.

Results from the PLS analysis revealed, NIR has a good potential for predicting physico-chemical properties of Camembert cheese and also can provide a fast and efficient method for continuously monitoring compositional changes and quality during cheese making in industries.

^{1}H NMR was found to be able to quicklyidentified and quantified 15 amino acids from Camembert cheese during 30 days of ripening. VIP results showed lysine and isoleucine are the most important amino acids during ripening of Camembert cheese. Discriminated results from ^{1}H NMR showed this method can be considered as a valid tool in food analysis which complements the traditional system of quality control.

The regression equation obtained by the following biomarkers as being the major contributors to Camembert cheese ripening: hexanoic acid, pentan-2-one, heptan-2-one, and heptan 2-ol, isoleucine and lysine, and also pH and a_w among physico-chemical properties.

This study has established a causative relationship for statistically important compounds, which is the first to characterize flavour and maturation profile of Camembert cheese by using chemometrics data to understand how aging chemistry related to flavour quality that is

developed during ripening. Furthermore, statistical methods made it possible to select a short list of potential biomarkers. These helpful methods could be especially beneficial for more accurate and in-depth research aimed at learning and observing the commitment of distinct and each flavour compounds to the maturation process of cheese varieties.

7.2 Limitations

Untargeted food metabolomics studies are limited is unidentified compounds are identified as being significant. In such cases time intensive isolation, purification and characterization must then be undertaken to identify said unknown compounds. The absence of libraries for many volatile and non-volatile constituents of food makes it a challenge to quickly identify. This obstacle needs to be overcome in order to obtain broader implementation of flavour assessment.

7.3 Future Work

Milk type (pasteurized or un pasteurized), season (winter or summer), storage of raw materials, processing parameters, and feed type of the source animal are the main sources of flavour variability in cheese products, which can all impact on final cheese flavour (Boltar et al., 2015). These reasons are why studying the flavour of cheese products is inherently difficult as I have shown here however, it is possible to generate useful, predictive models by combining multiple experimental and physical measurements.

Mathematical models of flavour release from soft cheeses could be carefully studied with more panels consuming a range of natural foods; allowing food industries to understand the efficiency and develop food flavours in their new products. Predictive models obtained for Camembert cheese could also be further verified with various seasonal products to make sure findings are repeatable and reproducible before routine use in the food industry could become a reality.

Chapter 8 REFERENCES

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APPENDICES

Appendix 1. PRIMER for ^{1}H NMR data set

RELATE

Testing matched resemblance matrices

Resemblance worksheet
Name: Resem1
Data type: Distance
Selection: All

Secondary data: Resemblance/model matrix

Resemblance worksheet
Name: Resem3
Data type: Distance
Selection: All

Parameters
Correlation method: Spearman rank

Sample statistic (Rho): 0.65
Significance level of sample statistic: 0.1 %
Number of permutations: 999
Number of permuted statistics greater than or equal to Rho: 0

BEST

Biota and/or Environment matching

Resemblance worksheet

Name: Resem1

Data type: Distance

Selection: All

Data worksheet

Name: Data3

Data type: Other

Sample selection: All

Variable selection: All

Parameters

Correlation method: Spearman rank

Method: BIOENV

Maximum number of variables: 5

Analyse between: Samples

Resemblance measure: D1 Euclidean distance

VARIABLES

pH Trial
aw Trial
Ts Trial
Mc Trial

Best result for each number of variables

No.Vars	Corr.	Selections
1	0.674	Ts
2	0.687	Ts,Mc
3	0.666	pH,Ts,Mc
4	0.650	pH,aw,Ts,Mc

Global Test

Sample statistic (Rho): 0.687

Significance level of sample statistic: 1%

Number of permutations: 99 (Random sample)

Number of permuted statistics greater than or equal to Rho: 0

Best results

No.Vars	Corr.	Selections
2	0.687	Ts,Mc
1	0.674	Ts
3	0.666	pH,Ts,Mc
2	0.663	pH,Ts
3	0.657	pH,aw,Ts
4	0.650	pH,aw,Ts,Mc
3	0.648	aw,Ts,Mc
1	0.638	Mc
2	0.630	pH,Mc
2	0.615	aw,Ts

DistLM

Distance based linear models

Resemblance worksheet

Name: Resem1

Data type: Distance

Selection: All

Transform: Log(X+1)

Resemblance: D1 Euclidean distance

Predictor variables worksheet

Name: Data3

Data type: Other

Sample selection: All

Variable selection: All

Transform: Log(X+1)

Selection criterion: AICc

Selection procedure: Step-wise

VARIABLES

1 pH Trial
2 aw Trial
3 Ts Trial
4 Mc Trial

Total SS(trace): 53.905

MARGINAL TESTS

Variable	SS(trace)	Pseudo-F	P	Prop.
pH	7.7814	3.7116	0.03	0.14435
aw	15.408	8.8049	0.003	0.28583
Ts	22.731	16.041	0.001	0.42168
Mc	22.26	15.475	0.001	0.41295

res.df: 22

NO STARTING TERMS

SEQUENTIAL TESTS

Variable	AICc	SS(trace)	Pseudo-F	P	Prop.	Cumul.	res.df
+Ts	10.848	22.731	16.041	0.001	0.42168	0.42168	22
+pH	6.4357	7.9264	7.16	0.001	0.14704	0.56873	21

BEST SOLUTION

AICc	R^2	RSS	No.Vars	Selections
6.4357	0.56873	23.248	2	1,3

Percentage of variation explained by individual axes

	% explained variation out of fitted model		% explained variation out of total variation	
Axis	Individual	Cumulative	Individual	Cumulative
1	74.62	74.62	42.44	42.44
2	25.38	100	14.44	56.87

dbRDA coordinate scores

Sample	dbRDA1	dbRDA2
curd	1.6456	-1.0967
curd	1.6703	-1.1471
curd	1.6168	-1.0457
cam1	0.99436	0.74815
cam1	0.99692	0.58872
cam1	0.98914	0.91436
cam5	0.80462	0.51112
cam5	0.8082	0.3609
cam5	0.79832	0.66736
cam10	0.063842	0.43429
cam10	0.065441	0.2869
cam10	0.05979	0.58746
cam15	-0.94749	0.1435
cam15	-0.94708	0.0064536
cam15	-0.95012	0.28553
cam20	-0.55702	-0.059784
cam20	-0.5542	-0.18998
cam20	-0.56231	0.074895
cam25	-0.80172	-0.25381
cam25	-0.79848	-0.3778
cam25	-0.80741	-0.12576
cam30	-1.195	-0.43889
cam30	-1.1919	-0.55724

```
 cam30   -1.2005   -0.31684
```

Relationships between dbRDA coordinate axes and orthonormal X variables

(multiple partial correlations)

```
Variable  dbRDA1  dbRDA2
Ts        -0.999   0.043
pH        -0.043  -0.999
```

Weights

(Coefficients for linear combinations of X's in the formation of dbRDA coordinates)

```
Variable    dbRDA1     dbRDA2
Ts         -8.2096  -0.011043
pH        -0.65972    -3.9064
```

Appendix 2. PRIMER for GC-MS data set

RELATE

Testing matched resemblance matrices

Resemblance worksheet
Name: Resem2
Data type: Distance
Selection: All

Secondary data: Resemblance/model matrix

Resemblance worksheet
Name: Resem3
Data type: Distance
Selection: All

Parameters
Correlation method: Spearman rank

Sample statistic (Rho): 0.694
Significance level of sample statistic: 0.1 %
Number of permutations: 999
Number of permuted statistics greater than or equal to Rho: 0

BEST

Biota and/or Environment matching

Resemblance worksheet

Name: Resem2

Data type: Distance

Selection: All

Data worksheet

Name: Data3

Data type: Other

Sample selection: All

Variable selection: All

Parameters

Correlation method: Spearman rank

Method: BIOENV

Maximum number of variables: 5

Analyse between: Samples

Resemblance measure: D1 Euclidean distance

VARIABLES

```
pH  pH Trial
aw  aw Trial
Ts  Ts Trial
Mc  Mc Trial
```

Best result for each number of variables

No.Vars	Corr.	Selections
1	0.726	Mc
2	0.713	aw,Mc
3	0.719	aw,Ts,Mc
4	0.694	pH,aw,Ts,Mc

Best results

No.Vars	Corr.	Selections
1	0.726	Mc
3	0.719	aw,Ts,Mc

```
2    0.713 aw,Mc
2    0.712 aw,Ts
2    0.699 Ts,Mc
4    0.694 pH,aw,Ts,Mc
3    0.685 pH,aw,Mc
3    0.681 pH,Ts,Mc
2    0.677 pH,Mc
1    0.655 Ts
```

DistLM

Distance based linear models

Resemblance worksheet
Name: Resem2
Data type: Distance
Selection: All
Transform: Log(X+1)
Resemblance: D1 Euclidean distance

Predictor variables worksheet
Name: Data3
Data type: Other
Sample selection: All
Variable selection: All
Transform: Log(X+1)

Selection criterion: AICc
Selection procedure: Step-wise

VARIABLES

1	pH Trial
2	aw Trial
3	Ts Trial
4	Mc Trial

Total SS(trace): 151.43

MARGINAL TESTS

Variable	SS(trace)	Pseudo-F	P	Prop.
pH	19.917	3.3319	0.034	0.13153
aw	69.711	18.767	0.001	0.46035
Ts	69.102	18.466	0.001	0.45633
Mc	70.318	19.072	0.001	0.46436

res.df: 22

NO STARTING TERMS

SEQUENTIAL TESTS

Variable	AICc	SS(trace)	Pseudo-F	P	Prop.	Cumul.	res.df
+Mc	33.798	70.318	19.072	0.001	0.46436	0.46436	22
+pH	22.898	34.952	15.901	0.001	0.23081	0.69517	21
+Ts	19.95	9.9898	5.5237	0.002	0.065969	0.76114	20

BEST SOLUTION

AICc	R^2	RSS	No.Vars	Selections
19.95	0.76114	36.171	3	1,3,4

Appendix 3. PLS Regression

C6 versus pH, aw, Ts, Mc

Method

Cross-validation	Leave-one-out
Components to evaluate	User specified
Number of components evaluated	4
Number of components selected	4

Analysis of Variance for C6

Source	DF	SS	MS	F	P
Regression	4	2602.48	650.619	1028.03	0.000
Residual Error	19	12.02	0.633		
Total	23	2614.50			

Model Selection and Validation for C6

Components	X Variance	Error	R-Sq	PRESS	R-Sq (pred)
1	0.66248	240.493	0.908016	281.556	0.892310
2	0.93739	75.208	0.971234	101.674	0.961111
3	0.98806	43.687	0.983291	61.216	0.976586
4	1.00000	12.025	0.995401	17.482	0.993313

X Loadings

	Comp1	Comp2	Comp3	Comp4
pH	-0.064417	0.960814	0.082729	0.085378
aw	-0.571299	-0.139651	0.930527	-0.309282
Ts	0.597335	-0.031220	0.568399	-0.793125
Mc	-0.584455	0.289718	-0.337774	-0.517694

Y Calculated

Row	C6
1	0.0586
2	-0.0892
3	-0.0647
4	1.2474
5	1.5555
6	1.3241
7	5.5595
8	5.8676
9	5.6362
10	8.5093
11	8.8174
12	8.5860
13	15.6739
14	15.9820
15	15.7506

16	19.0452
17	19.3532
18	19.1219
19	24.9425
20	25.2505
21	25.0192
22	30.1562
23	30.4642
24	30.2329

PLS Regression: time versus amino acids

Method

Cross-validation	Leave-one-out
Components to evaluate	User specified
Number of components evaluated	10
Number of components selected	7

Analysis of Variance for time

Source	DF	SS	MS	F	P
Regression	7	2604.40	372.057	589.45	0.000
Residual Error	16	10.10	0.631		
Total	23	2614.50			

Model Selection and Validation for time

Components	X Variance	Error	R-Sq	PRESS	R-Sq (pred)
1	0.459877	555.677	0.787463	727.178	0.721867
2	0.783414	45.284	0.982680	63.375	0.975760
3	0.895550	36.643	0.985985	52.624	0.979872
4	0.930834	17.913	0.993149	48.517	0.981443
5	0.954627	13.185	0.994957	34.773	0.986700
6	0.965319	10.666	0.995921	30.557	0.988312
7	0.976601	10.099	0.996137	28.492	0.989102
8		9.842	0.996236	31.505	0.987950
9		9.119	0.996512	44.293	0.983059
10		8.793	0.996637	58.093	0.977781

X Loadings

	Comp1	Comp2	Comp3	Comp4	Comp5	Comp6	Comp7
Aspartic acid	-0.389469	0.075949	0.103936	-0.204349	0.048895	0.050452	-0.308341
Aspargine	0.118248	0.321180	-0.373192	0.232246	-0.675838	0.750593	-0.542071
glutamic acid	-0.046443	0.373971	0.279725	-0.492156	-0.034677	0.033960	0.793298
Glycine	-0.378749	0.079058	0.251497	-0.283063	-0.018785	0.003739	0.085793
phenylalanine	-0.166894	0.380693	-0.043599	0.007342	0.190336	-0.504220	-0.151607
valine	-0.094283	0.411977	0.118248	0.058537	-0.101508	-0.375767	-0.051262
threonine	-0.375980	0.077310	0.277644	-0.267479	-0.085150	0.078490	-0.200780
serine	-0.368910	0.121268	-0.233991	0.061676	0.175257	0.114226	-0.324723
proline	-0.385850	0.088886	0.158808	-0.188683	0.057134	0.058071	-0.248610
methionine	-0.109392	0.343500	0.596527	0.070423	-0.197444	0.119566	-0.125383
lysine	-0.176758	0.285672	-0.610887	0.419786	0.099278	-0.265463	0.298370
leucine	0.177798	0.330908	0.087998	-0.389338	0.680728	0.087756	-0.322323
Histidine	-0.313354	0.183578	-0.410472	0.216561	0.337023	0.198770	0.074609
isoleucine	-0.361890	0.141908	-0.264782	0.216376	-0.212678	0.050482	0.081191
glutamine	-0.289360	0.125198	0.619951	-0.047266	-0.132026	0.213217	0.307750
lactate	-0.220399	0.159791	-0.783327	0.441811	-0.147325	-0.005302	0.316947

Y Calculated

Row	time
1	-0.6804
2	0.2586
3	0.6108
4	1.4813
5	0.6937
6	0.6371
7	4.6366
8	5.5068
9	4.4443
10	11.4333
11	10.2004
12	9.9615
13	15.5188
14	14.1377
15	15.1178
16	19.3067
17	20.3556
18	18.9366
19	25.3523
20	25.3100
21	25.4740
22	29.8389
23	30.8671
24	28.6007

PLS Regression: time versus Aspartic acid, Aspargine, ... amine, lactate

Method

Cross-validation	Leave-one-out
Components to evaluate	User specified
Number of components evaluated	10
Number of components selected	7

Analysis of Variance for time

Source	DF	SS	MS	F	P
Regression	7	2604.40	372.057	589.45	0.000
Residual Error	16	10.10	0.631		
Total	23	2614.50			

Model Selection and Validation for time

Components	X Variance	Error	R-Sq	PRESS	R-Sq (pred)
1	0.459877	555.677	0.787463	727.178	0.721867
2	0.783414	45.284	0.982680	63.375	0.975760
3	0.895550	36.643	0.985985	52.624	0.979872
4	0.930834	17.913	0.993149	48.517	0.981443

5	0.954627	13.185	0.994957	34.773	0.986700
6	0.965319	10.666	0.995921	30.557	0.988312
7	0.976601	10.099	0.996137	28.492	0.989102
8		9.842	0.996236	31.505	0.987950
9		9.119	0.996512	44.293	0.983059
10		8.793	0.996637	58.093	0.977781

X Loadings

	Comp1	Comp2	Comp3	Comp4	Comp5	Comp6	Comp7
Aspartic acid	-0.389469	0.075949	0.103936	-0.204349	0.048895	0.050452	-0.308341
Aspargine	0.118248	0.321180	-0.373192	0.232246	-0.675838	0.750593	-0.542071
glutamic acid	-0.046443	0.373971	0.279725	-0.492156	-0.034677	0.033960	0.793298
Glycine	-0.378749	0.079058	0.251497	-0.283063	-0.018785	0.003739	0.085793
phenylalanine	-0.166894	0.380693	-0.043599	0.007342	0.190336	-0.504220	-0.151607
valine	-0.094283	0.411977	0.118248	0.058537	-0.101508	-0.375767	-0.051262
threonine	-0.375980	0.077310	0.277644	-0.267479	-0.085150	0.078490	-0.200780
serine	-0.368910	0.121268	-0.233991	0.061676	0.175257	0.114226	-0.324723
proline	-0.385850	0.088886	0.158808	-0.188683	0.057134	0.058071	-0.248610
methionine	-0.109392	0.343500	0.596527	0.070423	-0.197444	0.119566	-0.125383
lysine	-0.176758	0.285672	-0.610887	0.419786	0.099278	-0.265463	0.298370
leucine	0.177798	0.330908	0.087998	-0.389338	0.680728	0.087756	-0.322323
Histidine	-0.313354	0.183578	-0.410472	0.216561	0.337023	0.198770	0.074609
isoleucine	-0.361890	0.141908	-0.264782	0.216376	-0.212678	0.050482	0.081191
glutamine	-0.289360	0.125198	0.619951	-0.047266	-0.132026	0.213217	0.307750
lactate	-0.220399	0.159791	-0.783327	0.441811	-0.147325	-0.005302	0.316947

Y Calculated

Row	time
1	-0.6804
2	0.2586
3	0.6108
4	1.4813
5	0.6937
6	0.6371
7	4.6366
8	5.5068
9	4.4443
10	11.4333
11	10.2004
12	9.9615
13	15.5188
14	14.1377
15	15.1178
16	19.3067
17	20.3556
18	18.9366
19	25.3523
20	25.3100
21	25.4740
22	29.8389
23	30.8671
24	28.6007

PLS Regression: Time versus Hexanoic acid, ... sine, Isoleucine, pH, aw

Method

Cross-validation	Leave-one-out
Components to evaluate	Adjusted
Number of components evaluated	8
Number of components selected	3

Analysis of Variance for Time

Source	DF	SS	MS	F	P
Regression	3	2523.36	841.119	184.57	0.000
Residual Error	20	91.14	4.557		
Total	23	2614.50			

Model Selection and Validation for Time

Components	X Variance	Error	R-Sq	PRESS	R-Sq (pred)
1	0.672601	291.837	0.888378	419.809	0.839431
2	0.742463	121.549	0.953510	206.025	0.921199
3	0.775665	91.144	0.965139	160.837	0.938483
4		89.712	0.965687	168.674	0.935485
5		85.950	0.967126	187.669	0.928220
6		85.165	0.967426	242.330	0.907313
7		85.130	0.967439	258.696	0.901053
8		85.122	0.967442	263.492	0.899219

Y Calculated

Row	Time
1	0.9517
2	0.6605
3	1.4741
4	1.1666
5	0.2073
6	2.8026
7	6.8050
8	6.2493
9	7.2293
10	8.6345
11	8.9631
12	12.0124
13	10.7735
14	11.9336
15	10.2120
16	20.5053
17	21.0884
18	20.2299
19	25.2980
20	26.0568

21	24.2704
22	28.7876
23	28.5388
24	33.1495

	Time	Acetic acid	Propanoic	2-Methyl	Butyric a	Isovaleric	Hexanoic	Octanoic a	Decanoic	Pentan-2-	Hexan-2-(	Undecan-	Nonan-2-(	Octan-2-o	4-Heptan	Heptan-2-	Acetoin	Acetophe	Acetone	Propanol	Heptanol	2-phenyle	Pentan-2-	Heptan-2-	2-Ethylhe	Nonan-2-(	3-Methylt	Ethanol	Butanol	Pinene	Decalacto	Butyrolac	Ethyl buta	Ethyl acet	Isoamyl fo	Amyl acet	Butyl buta	Ethyl form	Hexyl buta	Tetrahydr	Phellandr	Benzaldeh	Octanal	Nonanal	Decanal	Dodecana	Hexanal
Time	1																																												
Acetic acid	0.782364	1																																											
Propanoic acid	-0.39833	-0.46907	1																																											
2-Methyl propan	-0.52168	-0.54618	0.944502	1																																									
Butyric acid (but	0.801459	0.490753	-0.09249	-0.30121	1																																								
Isovaleric acid (	0.492944	0.487354	-0.33629	-0.46309	0.479542	1																																							
Hexanoic acid	0.90387	0.608588	-0.30651	-0.4868	0.937893	0.554867	1																																						
Octanoic acid	0.560005	0.219578	-0.32122	-0.2977	0.654858	0.197998	0.680844	1																																					
Decanoic acid	0.841207	0.512572	-0.13459	-0.33309	0.971201	0.43902	0.922913	0.647303	1																																				
Pentan-2-one	0.932275	0.601192	-0.25992	-0.42174	0.806951	0.465319	0.866115	0.496322	0.865025	1																																			
Hexan-2-one	0.833998	0.522769	-0.12994	-0.28234	0.953558	0.462603	0.94804	0.757325	0.904378	0.80731	1																																			
Undecan-2-one	0.416755	0.212306	-0.12339	-0.18308	0.374832	0.117286	0.346811	0.247311	0.5064	0.471593	0.262856	1																																		
Nonan-2-one	0.71317	0.387706	0.124139	-0.06611	0.877188	0.225321	0.784874	0.554398	0.931322	0.767837	0.794838	0.452007	1																																	
Octan-2-one	0.778528	0.469037	-0.12849	-0.29666	0.865965	0.343898	0.826469	0.620974	0.940767	0.791392	0.781195	0.472216	0.951718	1																															
4-Heptanone	0.814006	0.494443	-0.18981	-0.33767	0.75247	0.357605	0.775944	0.521072	0.859295	0.879842	0.706187	0.426677	0.862105	0.929145	1																														
Heptan-2-one	0.916217	0.565763	-0.21544	-0.38095	0.885983	0.415394	0.901988	0.615876	0.954879	0.944107	0.848143	0.51194	0.90532	0.942151	0.9402	1																														
Acetoin	-0.25159	-0.21239	-0.49283	-0.48911	-0.03782	-0.12921	-0.03391	0.213746	-0.06688	-0.24202	-0.09121	-0.11376	-0.18611	-0.08567	-0.18444	-0.18345	1																														
Acetophenone	-0.47595	-0.51351	0.98248	0.945323	-0.21512	-0.39776	-0.42112	-0.39874	-0.2413	-0.33416	-0.25251	-0.13355	0.024753	-0.21466	-0.25171	-0.30087	-0.49319	1																														
Acetone	-0.4798	-0.51786	0.988279	0.961186	-0.21686	-0.40098	-0.42453	-0.40197	-0.24325	-0.33686	-0.25455	-0.13463	0.026853	-0.21639	-0.25374	-0.30336	-0.49708	0.993076	1																													
Propanol	0.572194	0.363868	-0.00821	-0.19537	0.881654	0.41948	0.762967	0.547901	0.760063	0.53951	0.855758	0.072956	0.631437	0.576696	0.402585	0.598981	0.062769	-0.13367	-0.13476	1																												
Heptanol	-0.45697	-0.49323	0.925689	0.916331	-0.20654	-0.3819	-0.40433	-0.38284	-0.23168	-0.32084	-0.24244	-0.12823	0.027406	-0.2061	-0.24167	-0.28943	-0.47329	0.91551	0.950502	-0.12835	1																											
2-phenylethanol	0.061517	0.117383	-0.15939	-0.01842	-0.21052	-0.38925	-0.08408	0.033532	-0.23614	-0.19072	-0.09028	-0.1307	-0.25543	-0.21007	-0.24633	-0.16732	-0.16404	-0.13756	-0.13867	-0.13082	-0.13207	1																										
Pentan-2-ol	0.259485	-0.05593	-0.23584	-0.29597	0.199543	0.347019	0.303704	0.143316	0.141146	0.438604	0.285452	-0.04335	-0.04861	-0.04808	0.13927	0.20076	0.074071	-0.26101	-0.26312	0.213471	-0.2506	-0.25543	1																									
Heptan-2-ol	0.948586	0.723595	-0.28338	-0.4475	0.857515	0.596449	0.935655	0.537551	0.860109	0.883347	0.870237	0.287844	0.754566	0.813647	0.810975	0.90075	-0.27444	-0.38061	-0.38369	0.657151	-0.36544	-0.00771	0.242713	1																								
2-Ethylhexanol	-0.16778	-0.25453	0.985006	0.963315	-0.02565	-0.25902	-0.14273	-0.19556	-0.02808	-0.07114	-0.03006	-0.02756	0.149442	-0.00718	-0.00865	-0.04838	-0.47628	0.988274	0.988274	-0.03483	0.988274	-0.05482	-0.18804	-0.10288	1																							
Nonan-2-ol	0.57939	0.367859	-0.00766	-0.1977	0.889535	0.428261	0.77725	0.556308	0.767997	0.5458	0.866046	0.074852	0.640718	0.586341	0.411666	0.607013	0.062985	-0.13536	-0.13645	0.999275	-0.12996	-0.13246	0.214677	0.668883	-0.03331	1																						
3-Methylbutanol	0.718428	0.595981	-0.13367	-0.29992	0.883672	0.693008	0.863332	0.731615	0.84302	0.584814	0.872019	0.733634	0.74592	0.801169	0.630226	0.745145	-0.08024	-0.25799	-0.26018	0.815676	-0.24723	0.078249	0.001963	0.832353	-0.0812	0.831644	1																				
Ethanol	-0.4653	-0.50288	0.967189	0.958959	-0.21031	-0.38886	-0.4117	-0.38982	-0.2359	-0.32669	-0.24686	-0.13056	0.030565	-0.20986	-0.24608	-0.29384	-0.48186	0.943626	0.968025	-0.13068	0.917421	-0.13448	-0.25517	-0.3721	0.988274	-0.13233	-0.25194	1																		
Butanol	-0.46192	-0.49928	0.956551	0.954302	-0.20878	-0.38604	-0.40871	-0.38699	-0.23419	-0.32431	-0.24506	-0.12961	0.031197	-0.20833	-0.24429	-0.29182	-0.47831	0.926919	0.960327	-0.12974	0.938504	-0.1335	-0.25332	-0.3694	0.988274	-0.13137	-0.25003	0.996207	1																	
Pinene	-0.13749	0.109127	0.418493	0.504122	-0.12338	-0.37553	-0.17689	-0.10598	-0.13116	-0.23558	-0.07345	0.014564	0.035284	-0.09998	-0.17764	-0.18172	-0.27445	0.367288	0.409699	-0.11585	0.426437	0.270681	-0.53586	-0.12158	0.048506	-0.10933	0.036943	0.493073	0.506859	1															
Decalactone	0.744116	0.431325	-0.13808	-0.29465	0.808983	0.338495	0.780353	0.562879	0.899353	0.776674	0.712512	0.795421	0.85755	0.890458	0.817739	0.883982	-0.1045	-0.21279	-0.21452	0.48368	-0.20431	-0.20825	0.004351	0.718824	-0.00751	0.493663	0.880246	-0.20803	-0.20652	-0.0473	1													
Butyrolactone	-0.46721	-0.50461	0.975981	0.949031	-0.21117	-0.39046	-0.4134	-0.39142	-0.23687	-0.32803	-0.24787	-0.1311	0.027358	-0.21072	-0.24709	-0.29493	-0.48402	0.975072	0.974017	-0.13122	0.867511	-0.13503	-0.25622	-0.37363	0.988274	-0.13287	-0.25303	0.976301	0.955087	0.426043	-0.20889	1												
Ethyl butanoate	0.763063	0.463486	-0.12659	-0.29103	0.840408	0.334306	0.813157	0.609181	0.911208	0.774293	0.765319	0.397459	0.938609	0.992074	0.935375	0.926468	-0.08724	-0.21068	-0.21238	0.55094	-0.20228	-0.20617	-0.05087	0.811261	-0.00581	0.5613	0.810245	-0.20596	-0.20447	-0.10294	0.851736	-0.20681	1											
Ethyl acetate	0.012165	0.058986	-0.05793	0.137311	-0.23829	-0.4406	-0.13378	0.150909	-0.26729	-0.23578	-0.0684	-0.14794	-0.25227	-0.23778	-0.27882	-0.20166	-0.22333	-0.05793	-0.03624	-0.14807	-0.03341	0.953862	-0.28912	-0.06816	0.048826	-0.14994	0.019523	-0.02957	-0.02862	0.370123	-0.23572	-0.03313	-0.23337	1										
Isoamyl formate	0.16823	0.506112	-0.40785	-0.40328	-0.17814	0.454867	-0.07178	-0.33652	-0.07131	0.169544	-0.22006	0.205236	-0.1593	-0.04094	0.132802	0.040699	-0.13628	-0.35198	-0.35482	-0.33473	-0.33794	-0.34445	0.016864	0.087315	-0.2801	-0.33894	-0.25452	-0.3441	-0.3416	-0.08915	0.035912	-0.34552	-0.04584	-0.38989	1									
Amyl acetate	0.414921	0.236546	-0.16012	-0.18338	0.249958	-0.02174	0.247966	0.226946	0.442923	0.466949	0.144086	0.51167	0.563126	0.664862	0.730439	0.590813	-0.16518	-0.13818	-0.1393	-0.13141	-0.13267	-0.13523	-0.25659	0.342639	0.008036	-0.13306	-0.02592	-0.13509	-0.13411	-0.09608	0.615451	-0.13565	0.684291	-0.15307	0.270761	1							
Butyl butanoate	0.713287	0.490127	-0.28025	-0.41168	0.566206	0.288849	0.646488	0.339549	0.677321	0.835546	0.551977	0.572373	0.606079	0.643854	0.802609	0.756191	-0.06775	-0.31774	-0.32031	0.2124	-0.30507	-0.31095	0.347348	0.61559	-0.09746	0.222431	0.350894	-0.31063	-0.30838	-0.0742	0.73439	-0.31191	0.632056	-0.35197	0.334096	0.531981	1					
Ethyl formate	0.062379	0.11201	-0.16162	0.039262	-0.21347	-0.39471	-0.08872	0.21184	-0.23945	-0.20012	-0.03477	-0.13253	-0.25208	-0.21301	-0.24978	-0.16795	-0.16928	-0.13948	-0.14061	-0.13265	-0.13392	0.944279	-0.25901	-0.03109	-0.05482	-0.13432	0.078249	-0.13636	-0.13537	0.313304	-0.21116	-0.13692	-0.20906	0.992025	-0.34928	-0.13712	-0.31531	1					
Hexyl butanoate	-0.53622	-0.47994	0.912811	0.891581	-0.26437	-0.48882	-0.46878	-0.42526	-0.28719	-0.40105	-0.31031	-0.16412	-0.02041	-0.2638	-0.30933	-0.36838	-0.29688	0.908975	0.91153	-0.16428	0.799668	-0.16905	-0.32076	-0.46775	0.724464	-0.16634	-0.3211	0.938247	0.916755	0.533367	-0.26151	0.955472	-0.25891	-0.07259	-0.29974	-0.16982	-0.28848	-0.17142	1				
Tetrahydrofuran	-0.47783	-0.51599	0.988373	0.967281	-0.21597	-0.39333	-0.42279	-0.40032	-0.24226	-0.33549	-0.25351	-0.13408	0.028344	-0.21551	-0.2527	-0.30196	-0.49498	0.983346	0.99535	-0.1342	0.939846	-0.1381	-0.26204	-0.38212	0.988274	-0.13589	-0.25906	0.987317	0.9796	0.442044	-0.21364	0.985205	-0.21151	-0.03406	-0.35337	-0.13873	-0.319	-0.14004	0.931897	1			
Phellandrene	-0.47567	-0.51373	0.980308	0.966141	-0.215	-0.39753	-0.42088	-0.39851	-0.24116	-0.33397	-0.25236	-0.13347	0.029255	-0.21453	-0.25156	-0.3007	-0.49268	0.967843	0.990088	-0.1336	0.962899	-0.13748	-0.26086	-0.38039	0.988274	-0.13528	-0.25783	0.988317	0.988793	0.461311	-0.21267	0.966619	-0.21055	-0.03296	-0.35177	-0.1381	-0.31756	-0.1394	0.913613	0.995534	1		
Benzaldehyde	-0.22386	-0.15846	0.041363	0.296853	-0.36289	-0.28612	-0.29902	0.265999	-0.38411	-0.3215	-0.12373	-0.21667	-0.3841	-0.40272	-0.34271	-0.3551	-0.10415	0.078313	0.077083	-0.25454	0.064541	0.330931	-0.08656	-0.33877	0.190657	-0.25774	-0.51758	0.077597	0.074808	0.265659	-0.35821	0.081733	-0.39522	0.555924	-0.14377	-0.25492	-0.24945	0.570329	0.077744	0.078316	0.075674	1		
Octanal	-0.85413	-0.72467	0.773963	0.795533	-0.50233	-0.3706	-0.70639	-0.51433	-0.55104	-0.73236	-0.57789	-0.31186	-0.33971	-0.50125	-0.58776	-0.67066	-0.04954	0.807464	0.81032	-0.31215	0.753749	-0.26533	-0.3371	-0.72986	0.534636	-0.31607	-0.46705	0.791977	0.781734	0.272545	-0.4969	0.802802	-0.49195	-0.18241	-0.25106	-0.32267	-0.60636	-0.26568	0.820724	0.810287	0.802017	0.081168	1	
Nonanal	-0.93903	-0.87683	0.457036	0.528949	-0.57864	-0.44836	-0.732	-0.38134	-0.6319	-0.8309	-0.64914	-0.35913	-0.49932	-0.57772	-0.67657	-0.75702	0.352884	0.496088	0.508568	-0.35977	0.506392	-0.16411	-0.21532	-0.84254	0.212741	-0.36429	-0.55104	0.499117	0.502012	0.069189	-0.57244	0.485239	-0.56701	-0.12092	-0.37327	-0.3719	-0.66638	-0.17597	0.53855	0.507518	0.512339	0.096428	0.867539	1
Decanal	-0.83366	-0.64137	0.320267	0.406671	-0.53442	-0.45482	-0.66294	-0.26113	-0.57115	-0.76268	-0.56758	-0.33177	-0.45142	-0.53326	-0.62531	-0.70104	0.450196	0.369679	0.368114	-0.33208	0.346762	-0.21108	-0.28724	-0.7937	0.138326	-0.33626	-0.51865	0.345581	0.341132	0.254553	-0.52864	0.35495	-0.52337	-0.12751	-0.19232	-0.34328	-0.47953	-0.15784	0.504491	0.362527	0.358494	0.219042	0.740533	0.841701	1
Dodecanal	-0.80703	-0.79786	0.619876	0.632395	-0.4216	-0.56789	-0.61141	-0.33834	-0.45398	-0.63401	-0.49487	-0.26174	-0.26126	-0.42069	-0.4933	-0.57622	0.271184	0.652897	0.651592	-0.26198	0.602332	-0.26959	-0.17671	-0.74084	0.366746	-0.26527	-0.53778	0.628469	0.61861	0.191517	-0.41704	0.643532	-0.41289	-0.20744	-0.40264	-0.27081	-0.41574	-0.27336	0.732015	0.648623	0.63995	0.062447	0.848516	0.879053	0.857728	1
Hexanal	-0.84208	-0.44057	0.036691	0.200278	-0.76072	-0.22685	-0.77929	-0.42832	-0.82816	-0.93693	-0.74917	-0.46642	-0.81527	-0.76801	-0.85743	-0.90927	0.326798	0.11077	0.109012	-0.48772	0.10058	0.078037	-0.34036	-0.78763	-0.09128	-0.49377	-0.54117	0.100105	0.09805	0.128396	-0.75837	0.105226	-0.76816	0.123318	0.043767	-0.50437	-0.78162	0.118672	0.183954	0.106632	0.104574	0.321178	0.594324	0.713268	0.700401	0.4576	1